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Brooke Fahrig

Louisiana State University and Agricultural and Mechanical College

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CRYOPRESERVATION BY PELLET FREEZING
OF EPIDIDYMAL AND EJACULATED SPERMATOZOA
FROM MALE DOGS

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

In

The Interdepartmental Program of Animal and Dairy Sciences

by
Brooke M. Fahrig
B.S., Louisiana State University, 2000
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ABSTRACT

In this study, I evaluated the cryopreservation by pellet freezing of spermatozoa from individual dogs. In Experiment I, spermatozoa from 15 pairs of epididymides were suspended in glycerol, frozen as pellets of 10, 50, 100, or 200 μ l volumes, and thawed by dilution with TALP (Tyrode's solution plus albumin, lactate, pyruvate). In Experiment II, spermatozoa from 16 pairs of epididymides were suspended in glycerol, dimethyl sulfoxide, or ethylene glycol, frozen as 100 μ l pellets, and thawed by dilution with TALP, canine capacitation medium (CCM), or 3% sodium citrate solution. In Experiment III, spermatozoa from 15 pairs of epididymides were suspended in glycerol, frozen as 100 μ l pellets, and thawed by dilution with CCM. In Experiment IV, ejaculated spermatozoa from each of three dogs and epididymal spermatozoa from each of four other dogs were suspended in glycerol and were frozen and thawed as in Experiment III. Survival was determined by microscopic evaluation of motility and of membrane integrity. In Experiments III and IV, survival was also assayed by measuring the zona-binding capacity of the spermatozoa.

Survival of frozen-thawed samples was significantly lower than unfrozen samples. Sperm survival after freezing depended significantly on the pellet volume, reflecting the effect of cooling rate as a function of pellet volume. Thawing solutions and CPAs also significantly affected post-thaw sperm survival. The highest post-thaw survival was obtained with a pellet volume of 100 μ l and with the CPA-thawing solution combinations of glycerol-CCM and glycerol-TALP. The number of membrane-intact spermatozoa bound to each oocyte was

significantly higher for unfrozen samples than for frozen-thawed samples. Ejaculated spermatozoa exhibited survival and zona-binding capacity similar to that of epididymal spermatozoa, and there was little variation in the survival of ejaculated spermatozoa from an individual dog. There were significant differences in post-thaw sperm survival and zona-binding capacity among individual dogs. These results complement previous studies showing male-to-male differences in freezing susceptibility of canine spermatozoa.

CHAPTER 1

INTRODUCTION

Preservation of spermatozoa is an important tool to preserve genetic diversity and to assist in the reproduction of a species. The family Canidae has many representatives that may benefit from the use of semen preservation as a tool for assisting conservation (Goodrowe et al., 2000; Watson and Holt, 2001). At present, the gray wolf (*Canis lupus*), red wolf (*Canis rufus*), Mexican wolf (*Canis lupus baileyi*), African wild dog (*Lycaon pictus*), Ethiopian wolf (*Canis simensis*), South American savannah dog (*Speothos venaticus*), maned wolf (*Chrysaocoon brachyurus*), San Joaquin kit fox (*Vulpes macrotis*), and Northern swift fox (*Vulpes velox hebes*) are all considered to be threatened or endangered species (Farstad, 2000a). The goal of this project was to derive a method by which canine epididymal spermatozoa might be frozen easily and efficiently yet exhibit high survival when thawed. To accomplish this, I attempted to optimize the steps that influence the survival and fertilizing capacity of canine epididymal spermatozoa after being frozen as small volumes at rapid rates on dry ice.

The current status of cryopreservation of dog spermatozoa is puzzling. The first pregnancies from cryopreserved canine spermatozoa were produced over 30 years ago from semen frozen as pellets on dry ice (Seager, 1969), and artificial insemination of domestic bitches with frozen-thawed semen is now offered as a routine clinical service by many veterinarians. However, resultant pregnancy rates are highly variable and are generally lower than those with fresh semen (Linde-Forsberg, 1991). There are many variables that apparently

influence functional survival of cryopreserved dog spermatozoa. To complicate the derivation of efficacious methods of cryopreservation, spermatozoa from different individuals may exhibit significantly different responses to the same freezing treatments (Yu et al., 2002).

Spermatozoa from different species may exhibit significantly different responses to the same cryopreservation treatments and spermatozoa from different individuals within a species may also exhibit significant differences. Variations have been noted between the freezability of spermatozoa of different stallions, boars, and humans (Amann and Pickett, 1987; Cochran et al., 1983; Heuchel et al., 1983; Johnson et al., 1981; Larsson et al., 1976). Similar differences have also been noted among different dogs, but the extent of such differences is unknown (England, 1993). Yu et al. (2002) found that there were clear male-to-male differences when they froze and thawed epididymal spermatozoa from different dogs.

While there have been many studies of the freezing of ejaculated dog semen, few have been reported for epididymal spermatozoa. In this study, I have used epididymal, rather than ejaculated, spermatozoa to study the effects of pellet volumes, cryoprotective agents, and thawing solutions on canine spermatozoa for several reasons. I have attempted to devise procedures that might be used under field conditions for endangered and threatened species of canids. Recovery and cryopreservation of their epididymal spermatozoa would be one useful way to rescue the germplasm of specimens of endangered species that might die of natural causes, accidents, or poaching. Although I recognize

that there will be differences among canid species, my intention is to optimize principal variables of cryopreservation that may affect functional survival of spermatozoa of domestic and exotic canid species (Yu et al., 2002).

There are many variables that affect the fertility and survival of mammalian spermatozoa when cryopreserved (see reviews by Holt, 2000a; Leibo and Bradley, 1999; Parks, 1997; Watson, 1990,1995). Cooling rate is a very important variable, and when spermatozoa are frozen as pellets on dry ice, different volumes are likely to cool at different rates. When frozen at various cooling rates, cells of various types exhibit an inverted V-shaped survival curve (Mazur et al., 1972, 1984). Mazur and his colleagues hypothesized that such a survival curve can be interpreted as resulting from the interaction of two factors oppositely dependent on cooling rate. One factor results from the changing properties of the extracellular solution as water is removed in the form of ice. The second factor is intracellular ice formation and results from cells becoming increasingly supercooled with decreasing temperature. At high cooling rates, since the cells cannot lose water quickly enough to remain in osmotic equilibrium with extracellular solution, the likelihood of intracellular ice formation increases with increasing cooling rate. The rationale of Experiment I of this study was the following: canine spermatozoa have been successfully frozen as pellets. Furthermore, sperm survival depends on cooling rate. It was hypothesized that the size of droplets of sperm suspensions cooled on dry ice would influence their cooling rate. One goal of this investigation was to test that hypothesis. Therefore, the purpose of Experiment I was to determine whether the volumes of

droplets of epididymal spermatozoa from individual dogs frozen on dry ice would influence post-thaw spermatozoa survival.

Cryoprotectant additives and thawing solutions are also important considerations for cryopreservation of spermatozoa. In most species, glycerol has been the most commonly used cryoprotectant, although dimethyl sulfoxide has been used alone or in combination with glycerol (England, 1993). Usually, pelleted semen is thawed in a solution of saline or sodium citrate at 37°C (Lindforsberg, 1991). Spermatozoa frozen by the pellet technique can be thawed by direct immersion of the pellet into a warm solution, which causes the pellet to thaw rapidly while simultaneously reducing the cryoprotectant concentration. The purpose of Experiment II was to determine whether various combinations of CPAs and thawing solutions have an effect on post-thaw survival of epididymal spermatozoa from individual dogs.

Zona and oocyte penetration assays have been used successfully to estimate whether stored spermatozoa of various species, including canids, have acceptable fertilizing potential (Gadea and Matas, 2000; Gadea et al., 1998; Goodrowe et al., 2001; Ivanova et al., 1999; Mastro Monaco et al., 2002). The purpose of Experiment III was to determine the capacity of epididymal spermatozoa from individual dogs to bind to canine zonae prior to pellet-cryopreservation and after thawing.

The physiological properties of ejaculated spermatozoa differ from those of epididymal spermatozoa. I have used ejaculated spermatozoa in Experiment IV to determine if the method of pellet-cryopreservation that I have found to

produce the highest survival of epididymal spermatozoa can be used to cryopreserve ejaculated spermatozoa. The purpose of Experiment IV was to determine the capacity of ejaculated spermatozoa from individual dogs to bind to canine zonae prior to pellet-cryopreservation and after thawing compared to that of epididymal spermatozoa and to determine the reproducibility of the post-thaw survival of spermatozoa within an individual dog.

The overall purposes of this study were (1) to determine the effects of pellet volumes, cryoprotective agents, and thawing solutions on post-thaw survival of epididymal spermatozoa from individual dogs and (2) to determine the zona-binding capacity of epididymal spermatozoa from individual dogs prior to pellet-freezing and after thawing. Using the method found to produce the highest survival for epididymal spermatozoa, canine ejaculated spermatozoa was also cryopreserved to compare post-thaw survival and zona-binding capacity of the two categories of spermatozoa.

CHAPTER 2

LITERATURE REVIEW

Physiology of Spermatozoa

The male reproductive system consists of the testis, epididymis, vas deferens, accessory sex glands, and the penis. The testis produces spermatozoa and testosterone, as well as other substances such as inhibin, estrogen, and several proteins. The epididymis provides the environment for final maturation of spermatozoa and serves as a storage organ for these cells. The accessory sex glands produce seminal plasma, and the penis is the copulatory organ (Senger, 1999).

The testis has both gametogenic and endocrine functions (Mortimer, 1994). These two functions are closely related because an adequate level of androgen production is necessary for the production of spermatozoa and because the successful delivery of spermatozoa depends on normal sexual behavior and the development of secondary sexual characteristics, which are also under the control of androgens (Setchell, 1978). The bulk of the testis is comprised of the seminiferous tubules, which are looped tubules open at both ends, one end opening into the rete testis and the other into the epididymis. The seminiferous tubules lie within the lobes of the testis separated by fibrous septa and are enclosed by loose connective tissue containing blood vessels, lymphatics, nerves, and Leydig cells. The wall of each seminiferous tubule is primarily fibrous tissue with some myoid cells, giving it limited contractility (Mortimer, 1994).

The seminiferous tubule in the adult male contains a stratified epithelium of several layers of cells of widely different morphology and an irregular lumen. The seminiferous epithelium is made up of the Sertoli cells that extend from the basement membrane of the tubule wall to the tubule lumen. The various germinal cells are embedded between the Sertoli cells, which regulate their development. The germ cells go through a complicated series of divisions and maturational changes that together constitute the process of spermatogenesis, which produces haploid spermatids. The final transformation of spermatid into spermatozoon is known as spermiogenesis (Setchell, 1978).

The most immature male germinal cell is the spermatogonium. It is the basic self-renewing stem cell of the male germ cell line. Spermatogonia first undergo mitotic division to increase their number with daughter cells proceeding into spermatogenesis, forming replacement stem cells, or degenerating. Spermatogonia are the precursors of primary spermatocytes, into which they are transformed before entering the first meiotic division to produce secondary spermatocytes. The secondary spermatocytes go through the second meiotic division to produce haploid spermatids (Ibach et al., 1976).

Spermiogenesis is the complex maturational process by which a haploid spermatid differentiates into a mature spermatozoon. During this process, the nuclear histones are replaced by protamines, the chromatin condenses, and the smaller nucleus assumes an eccentric position close to the cell membrane. Residual cytoplasm is shed from the neck region of the mature spermatozoon as it is released from the seminiferous epithelium into the tubule lumen, a process

termed spermiation. A small residual cytoplasmic droplet also remains attached to testicular spermatozoa at the point of separation. As the cell undergoes further maturation during epididymal transit, the cytoplasmic droplet migrates along the tail and is finally lost (Senger, 1999).

In the dog, the epididymis lies along the dorso-lateral border of each testis. It is comprised of the vasa efferentia, which emanate from the rete testis, and the epididymal ducts. The epididymis opens into the vas deferens, which then passes through the inguinal canal into the peritoneal cavity and opens into the urethra adjacent to the prostate. The primary functions of the epididymis are post-testicular maturation and storage of spermatozoa during their passage from the testis to the vas deferens. In most mammalian species, sperm transport through the epididymis takes 10 to 15 days (Turner, 1979), with differences among species. The epididymal epithelium, which is androgen-dependent, has absorptive and secretory functions, and the epididymis is divided into three functionally distinct regions: caput epididymis, corpus epididymis, and cauda epididymis. Their functions can be described simplistically as concentration, maturation, and storage of spermatozoa, respectively (Amann et al., 1993).

Post-testicular sperm maturation involves an intricate combination of morphological, biochemical, biophysical, and metabolic changes. Physiologically, the spermatozoa develop motility as they traverse the epididymis (Hoskins et al., 1978). There is a correlation between the amount of free calcium surrounding the spermatozoon within the cauda epididymidis of a given species and level of sperm motility therein (Morton et al., 1978). The most striking

morphological change involves loss of the cytoplasmic droplet. Metabolically, the spermatozoa become more active and show increased fructolysis. Spermatozoa taken from the caput epididymidis are generally incapable of fertilization, whereas those from the distal corpus and the cauda are able to undergo capacitation and achieve normal gamete interaction (Mortimer, 1994).

As many as half of the spermatozoa released from the testis die and disintegrate within the epididymis and are resorbed by the epididymal epithelium. The remaining mature spermatozoa are stored in the cauda epididymides, which contain about 70% of all the spermatozoa present in the male tract (Mortimer, 1994). Although the environment of the cauda is adapted for sperm storage, the spermatozoa within it do not remain in a viable state indefinitely. After prolonged sexual inactivity, caudal spermatozoa first lose their fertilizing ability, followed by their motility, and they then disintegrate. Caudal spermatozoa are also sensitive to increased testicular temperature, which causes deterioration in sperm quality (Mortimer, 1994).

Bateman et al. (2000) characterized canine epididymal spermatozoa and examined the effects of age and animal size. Mean epididymal sperm characteristics were: motility, 75%; membrane integrity, 70%; and concentration, 200 to 300 × 10⁶ sperm/ml. Concentration was the only characteristic affected by dog age; dogs 6 to 7 months old had a lower sperm count compared to all other ages. No differences in sperm characteristics were observed between large and small breeds. Yu and Leibo (2002) found that motility was independent of age while membrane integrity in younger dogs was somewhat lower. They also

observed that spermatozoa collected from dogs that were one to five years old appeared to show higher percentages of spermatozoa capable of binding to canine zonae than dogs older than 6 years, although the numbers of spermatozoa that bound to zonae were highly variable.

Testicular spermatozoa of mammals are immotile, which is apparently due to the immaturity of the sperm plasma membrane. During passage through the epididymis, spermatozoa undergo substantial maturational changes that result in their acquisition of motility. Although motile sperm recovered from the cauda are capable of progressive motility when suspended in culture medium or diluent, they remain essentially immotile in vivo. Only at ejaculation, when they are mixed with secretions of the accessory glands, do sperm become motile (Mortimer, 1994).

At ejaculation, spermatozoa are abruptly transferred from storage in the caudae epididymides, mixed with the secretions of the accessory sex glands in a precise sequence, and emitted to the exterior along the penile urethra that has been prepared by the bulbourethral gland's secretion (Senger, 1999). In studies of the characteristics of ejaculated spermatozoa, Morton and Bruce (1989) found that fertile dogs siring a litter within the previous 12 months had the following mean semen characteristics: membrane integrity, 88%; normal morphology, 70%; total spermatozoa per ejaculate, 900×10^6 .

A normal, mature spermatozoon is typically described as possessing an oval head and a tail, which is a self-powered flagellum. The head is composed of the nucleus, the acrosome, and a post-nuclear cap. The anterior two-thirds of

the head, and hence the nucleus, is covered by the acrosome, which is a membrane-bound lysosome that contains hydrolytic enzymes. The tail is composed of the capitulum, the middle piece, the principal piece, and the terminal piece (Senger, 1999).

Mammalian spermatozoa cannot penetrate the zona pellucida immediately after ejaculation. According to Austin (1951) and Chang (1951), as cited by Barros (1973), a final stage of maturation, termed capacitation, is defined as the process by which spermatozoa acquire the capacity to undergo the acrosome reaction and fertilize ova. It is essential for fertilization both in vivo and in vitro. In all mammals, a marked change in sperm motility is associated with capacitation. An extremely vigorous but nonprogressive pattern, termed hyperactivated motility, develops as a result of a calcium influx and also binding to the uterine endometrium, causing increased flagellar curvature and hence extreme lateral movement of the sperm head. Hyperactivated motility is essential for the fertilization of intact oocyte-cumulus complexes in vitro and in vivo (Pollard et al., 1991).

After being capacitated, but normally only when in contact with an oocyte, spermatozoa are ready to undergo the acrosome reaction, an exocytotic event involving localized fusions between the outer acrosomal and sperm plasma membranes that result in formation of vesicles. The acrosomal contents, mainly the enzymes hyaluronidase and acrosin, are then released through holes in the vesicles. The enzymes released during the acrosome reaction facilitate penetration by the spermatozoon through the cumulus cells and the zona

pellucida. The acrosome reaction is essential for mammalian fertilization in vivo (Bedford, 1974).

Sperm penetration through the zona pellucida into the perivitelline space is primarily a mechanical process, aided by the hyperactivated motility pattern and by acrosin in softening the zona matrix. Emerging from the inner face of the zona pellucida, the fertilizing spermatozoon now enters the perivitelline space surrounding the oocyte. At this stage, the spermatozoon has completed the acrosome reaction and the inner acrosomal membrane is revealed over the anterior cap region of the sperm head and the equatorial segment remains intact. After attachment to the oolemma, the spermatozoon ceases its motility. Fusion is then initiated between the oolemma and the equatorial segment, leading to complete engulfment of the spermatozoon by the oocyte. As the sperm head is incorporated into the ooplasm, the chromatin with its nucleus undergoes decondensation. A new nuclear envelope is formed from components derived from the nucleus, and the male pronucleus is formed. At the moment of sperm-oocyte contact, the oocyte initiates the cortical reaction, which is the basis of the block to polyspermy. The male and female pronuclei move toward each other, and their nuclear envelopes break down. The chromosomes from the male and female gametes are released into the ooplasm, and the first cell division is initiated. This intermingling of parental genetic material is the completion of syngamy, the creation of a genetically new individual (Bedford, 1970, 1974; Mortimer, 1994).

Cryobiology of Spermatozoa

Cryopreservation is an integral part of assisted reproduction in many species. The principal variables of cryobiology derived from the study of animal gametes are: cooling and warming rates, developmental stage and species, intracellular ice formation, cell volume excursions during cooling, osmotic responses, temperature, and chilling injury (Leibo, 2002; Mazur et al., 1972, 1984). It has been observed that cells of various types exhibit an inverted V-shaped survival curve when frozen at various cooling rates (Mazur et al., 1972, 1984). Mazur and his colleagues hypothesized that such a survival curve can be interpreted as resulting from the interaction of two factors oppositely dependent on cooling rate. One factor results from the removal of water from the extracellular solution in the form of ice as the solution gradually freezes at decreasing subzero temperatures. The second factor results from cells becoming increasingly supercooled with decreasing temperature since they may not lose water quickly enough to remain in osmotic equilibrium with the extracellular solution. Mazur (1963) hypothesized that the survival of various cells subjected to low temperature exposure is higher when they are cooled slowly because slow cooling decreases the probability of intracellular freezing by permitting water to leave the cell rapidly enough to keep the protoplasm at its freezing point. It has been suggested that an intermediate cooling rate exists at which the damaging effects of these two factors are minimized (Holt, 2000b).

Cells may be damaged when subjected to osmotic shock. Such a shock can occur when cells suspended in a hypertonic solution are abruptly diluted into

an isotonic solution. An analogous dilution may occur during rapid thawing of a frozen solution. When aqueous solutions are frozen, water is removed in the form of ice causing a concomitant increase in the concentration of dissolved solutes. When the frozen solution is thawed, the ice melts, causing dilution of the concentrated solutes. Spermatozoa have been shown to be especially susceptible to osmotic shock (Leibo and Bradley, 1999).

The expression “cold shock” is commonly used to refer to the sensitivity of spermatozoa to sudden cooling. Spermatozoa demonstrate a loss of membrane integrity and cell function upon being rapidly cooled to a temperature within the range of 0 to 20°C. This phenomenon is probably related to phase transitions of membrane lipids resulting in phase separations and loss of selective permeability characteristics of living biological membranes. Egg yolk, the most effective agent to protect spermatozoa against cold shock, is commonly included in cryopreservation diluents (Watson, 1995).

Although effective cryopreservation procedures have been reported for spermatozoa of a number of species, mammalian spermatozoa provide the best example of the limits to predictive cryobiology. The general properties of spermatozoa should make them amenable to successful cryopreservation. They are small cells with high permeability to water and cryoprotectants, and they have low water content (Rall, 2001). However, practical experience indicates that the special properties of spermatozoa of many species make them especially difficult to cryopreserve (Holt, 2000b). First, sperm are highly differentiated cells with structural and functional compartmentalization. There is some evidence of

differential sensitivity of these compartments and associated cell membranes to osmotic and toxic stresses during cryopreservation (Henry et al., 1993; Thomas et al., 1998). Second, spermatozoa of many species are apparently injured when rapidly cooled to temperatures within the range of 0 to 20°C. Prior incubation with special additives, such as egg yolk, and slow cooling may reduce injury. Other complicating factors include heterogeneity in the properties of individual spermatozoa in a suspension, the unknown functions of seminal plasma components, and the need to preserve motility and associated metabolism. Differences among spermatozoa of different species in their tolerance to glycerol and other cryoprotective agents can be substantial and further complicate the matter (Holt, 2000a,b). These special properties often vary among species and even between individuals within a species (Rall, 2001). Differences among males in their sperm freezing sensitivity are found almost universally among various species (Leibo and Bradley, 1999).

When cells are frozen and thawed, they experience several cycles of dehydration and rehydration, resulting in extreme volume changes. The first volume change occurs when the cells are placed in a cryoprotective agent, such as glycerol. The volume changes once again when the cells are frozen, when they are thawed and when the cryoprotective agent is removed. When cell suspensions are frozen, they are cooled at finite rates often referred to as “slow” or “fast”. It is important to note that “slow”, “intermediate”, and “fast” are relative terms; a rate that is slow for one cell type may be fast for a second cell type. The optimum cooling rate is the rate at which maximum survival of cells occurs.

Efforts to determine mechanisms responsible for injury to spermatozoa have increased substantially in recent years (Gao et al., 1993, 1997; Watson, 1995). No single cryoprotective agent protects spermatozoa of all species, and there is not a single optimal cooling and a single optimal warming rate for spermatozoa of all species (Leibo and Bradley, 1999). Variations have been noted among the freezing susceptibilities of spermatozoa of individual stallions, boars, and humans (Amann and Pickett, 1987; Cochran et al., 1983; Heuchel et al., 1983; Johnson et al., 1981; Larsson et al., 1976). Similar effects have been noted in the dog, but the extent of this variation in ability to survive freezing is unknown (England, 1993). Breed influences have been noted in post-thaw fertility of boar semen (Johnson et al., 1981), and a similar relationship has been noted in the dog (Linde-Forsberg et al., 1999).

Yu et al. (2002) found that there were clear male-to-male differences when they froze and thawed epididymal spermatozoa from different dogs. This was very evident when post-thaw survival for each dog's spermatozoa was calculated as a percentage of the pre-freeze value. The values of post-thaw motility ranged from 40 to 100%. Another study of cryopreservation of spermatozoa from seven dogs found that post-thaw survival, calculated as a percentage of initial motility, ranged from 24 to 54% (Thomas et al., 1993). Ivanova et al. (1999) attempted to predict functional viability of cryopreserved spermatozoa from three dogs by sperm zona-binding capacity and found that post-thaw progressive motility varied from 35 to 55% and that the respective binding indices were 10, 17 and 27.

Songsasen et al. (2002) have also found male-to-male differences in the post-thaw survival of ejaculated canine spermatozoa.

Methods of Cryopreservation of Spermatozoa

Polge et al. (1949) reported that revival of fowl spermatozoa after vitrification and dehydration at low temperatures was possible. They then discovered that glycerol could be successfully used as a cryoprotective agent to freeze bovine and human spermatozoa. Luyet and Keane (1955) utilized a high velocity method of cryopreservation to freeze bull semen. A test tube containing the sperm suspension was corked and placed in a water bath at 0°C for 5 min. It was then frozen by abrupt immersion in an alcohol bath at the desired temperature and was then immersed in liquid nitrogen (LN₂). They found that bull semen could be successfully cooled from 0°C to -195°C in two abrupt immersions, one in an alcohol bath at -27°C and the other in LN₂. Nagase and Niwa (1964) later devised a method to freeze bull spermatozoa as pellets on dry ice. They did this by allowing small droplets of semen to fall directly into small depressions on the surface of a block of dry ice. The size of the pellets varied between 13 and 200 µl. Survival of spermatozoa using this method was found to be higher compared with the previously established two-step method of Luyet and Keane (1955).

After freezing ram spermatozoa as pellets, Salamon (1968) found that freezing diluted semen as pellets on dry ice or freezing it in ampules at a slow rate yielded higher survival compared to freezing spermatozoa in straws. In a later study, Salamon (1971) also found that ram spermatozoa frozen as pellets at

–79°C and –140°C showed similar survivals after thawing and that resultant lambing rates following insemination of ewes with the sperm frozen at the two temperatures were also comparable. Pursel and Johnson (1975) used the pellet method to cryopreserve boar spermatozoa. The pregnancy rate that they achieved by AI (artificial insemination) of gilts with pellet-frozen semen approached that of fresh semen. Graham et al. (1982) used the pellet method to freeze turkey spermatozoa on dry ice, which exhibited acceptable motility after being thawed.

In 1969, Seager had adapted the pellet-freezing method of Nagase and Niwa to dog sperm, and the first canine pregnancies resulted from artificial insemination of bitches with cryopreserved spermatozoa that had been frozen as pellets on dry ice. Within 4 years, this method had been used to produce 21 litters of pups from frozen-thawed semen, some of which had been stored in LN₂ for > 1 year (Seager and Fletcher, 1973). The efficiency of this method was demonstrated by the fact that the bitches inseminated by natural mating or by artificial insemination with fresh or with frozen semen had pregnancy rates of 78, 65, and 66%, respectively (Seager et al., 1975). There have been many studies of the freezing of ejaculated semen from dogs but few have been reported for epididymal spermatozoa. Since there are significant differences in the physiological characteristics of epididymal and ejaculated spermatozoa, it seems appropriate to determine whether the two categories of spermatozoa respond differently to cryopreservation.

The derivation of freezing protocols for dog spermatozoa has generally evolved in an empirical manner. A variety of compounds have been used as cryoprotective agents during freezing. These agents can be assigned to two categories – those that permeate cells (e.g., glycerol, dimethyl sulfoxide, ethylene glycol) and those that remain extracellular (e.g., proteins, sugars, synthetic macromolecules). For spermatozoa of most species, glycerol has been the most commonly used cryoprotectant, although dimethyl sulfoxide has been used alone or in combination with glycerol (Holt, 2000a). The optimal glycerol concentration varies with diluent, cooling method, and species. The optimum cryoprotective concentration depends on cooling rate as well; lower concentrations require faster cooling rates. In the dog, glycerol concentrations have varied between 4 and 11% (v/v) depending on diluent composition (England, 1993).

Egg yolk has been shown to protect cell membranes from cold shock and is regularly incorporated into diluents for spermatozoa (Holt, 2000a). The concentration of egg yolk used varies among species but is commonly used at concentrations of 3 to 25% (w/v) (England, 1993). For the preservation of dog spermatozoa, a concentration of 20% egg yolk has been used in several studies (Andersen, 1972; Linde-Forsberg and Forsberg, 1989). A lactose solution with egg yolk and glycerol was widely used for the pellet method of freezing semen from several species, and this method was successfully used in the dog (Andersen, 1972; Seager, 1969; Seager and Fletcher, 1975).

Although Seager and Fletcher (1973) indicated that survival of spermatozoa was similar following freezing in either 0.25 or 0.5 ml straws or pellets, Seager et al. (1975) subsequently utilized the pellet method, describing better survival than that following freezing in straws.

Canine spermatozoa are now frozen using many different extenders and methods. It is generally conceded that any successful spermatozoa freezing extender must contain glycerol as a cryoprotectant but high levels of glycerol seem to have a negative impact on fertility (Linde-Forsberg, 1991). Today, most facilities that cryopreserve canine spermatozoa use 0.5 ml straws and store the frozen specimens in LN₂. Some clinics still use the pellet method to freeze small droplets of spermatozoa on dry ice and then store them in liquid nitrogen. Overall, motility rates are usually somewhat lower after AI with frozen-thawed semen than with fresh or chilled semen (Brown, 1992).

While post-thaw motility of 40% or greater is desirable, Linde-Forsberg (1991) reported that pregnancies have been achieved by insemination of bitches with semen that exhibited only 20% motility. Farstad (1996) reported that the highest rates of cell survival after freezing and thawing require that the rate chosen for freezing is paired with an appropriate rate of thawing. Generally, it is believed that fast freezing requires fast thawing to reverse the osmotic balance, rehydrate and restore the lipo-protein configuration of the membrane in a fashion similar to the events induced by freezing. Usually, pelleted canine semen is thawed in a solution of saline or sodium citrate at 37°C (Linde-Forsberg, 1991). Spermatozoa frozen by the pellet technique can be thawed by direct immersion

of the pellets into a solution, which causes them to thaw rapidly while simultaneously reducing the cryoprotectant concentration.

Thomas et al. (1993) reported that freezing canine semen as pellets resulted in better post-thaw progressive motility than freezing it in 0.5 or 0.25 ml straws regardless of the diluent used. Olar et al. (1989) studied the influence of diluents, cryoprotectants, and spermatozoal processing procedures on post-thaw motility of canine spermatozoa frozen in straws. They found that post-thaw motility was highest when the diluent was egg yolk-Tris containing 2 to 4% glycerol. Frozen-thawed spermatozoa appeared to tolerate a range of glycerol concentrations, and the optimal glycerol concentration depended on the type of diluent used. Post-thaw motility increased as warming rate increased. Motility was highest when specimens were thawed in a 75°C water bath for 12 sec.

Dobrinski et al. (1993) reported on the effects of four diluents and three cooling rates on post-thaw motility of dog spermatozoa. Spermatozoa were diluted with one of four diluents and were then frozen in 0.5 ml straws at a low, intermediate, or high cooling rate. There was no difference among diluents as to the resultant progressive motility. The low, intermediate, and high cooling rates resulted in the highest, intermediate, and lowest motility values, respectively. All four diluents were found to be equally suitable for freezing canine spermatozoa at a low cooling rate.

Fontbonne and Badinand (1993) also studied the effect of glycerol on motility of canine spermatozoa after thawing. The ejaculates were frozen by methods differing in the following factors: the temperature at which glycerol was

added (5°C or 22°C); method of adding glycerol (in one or several steps); and the concentration of glycerol (1.6, 3.2, or 6.4%). There was no difference in progressive motility after thawing when the spermatozoa had been frozen in diluents containing 3.2 or 6.4% glycerol. Motility was lower when the extender contained only 1.6% glycerol. There were no significant differences among the results achieved regardless of the method used to add glycerol or the temperature at which it was added.

Hay et al. (1997a) also studied cryopreservation of canine spermatozoa. Their results suggest that the optimal cooling rate for freezing canine spermatozoa suspended in 4% glycerol is approximately 30°C/min. Spermatozoa frozen at higher and lower rates showed reduced motility and greater acrosomal damage.

Peña et al. (1998a) evaluated the effects of four concentrations of glycerol added to Tris-fructose-citric acid extender at 37°C or 4°C on dog sperm by monitoring post-thaw sperm longevity and acrosomal integrity. After being thawed, sperm motility and acrosomal integrity were highest when 8% glycerol was used in the diluent. When Peña et al. (1998a) compared the addition of glycerol at 37°C or 4°C, after being thawed, sperm motility and acrosomal integrity were not significantly different between treatments.

In a related study, Peña et al. (1998b) attempted to improve the efficiency of egg yolk as a diluent by the use of compounds containing sodium dodecyl sulfate (SDS) since this detergent has been found to be beneficial. They examined the longevity and membrane integrity of frozen-thawed dog

spermatozoa processed in an egg yolk diluent containing several different concentrations of SDS. It was found that longevity and membrane integrity of thawed spermatozoa were increased when 0.25% SDS was added to the diluent.

Rota et al. (1998) also investigated the survival of dog spermatozoa frozen in different glycerol concentrations and at different cooling and warming rates. They tested the effects of different combinations of 3 or 5% glycerol, cooling rates of 10°C/min or 50°C/min, and warming in water baths at 38°C for 1 min or at 70°C for 8 sec. The higher glycerol concentration and the higher temperature used for thawing produced a higher percentage of membrane-intact and motile spermatozoa during incubation, whereas, there were no differences between cooling rates.

Peña and Linde-Forsberg (2000a) studied the effects on post-thaw survival of dog sperm of adding Equex STM Paste, a substance containing the detergent SDS, to a TRIS-egg yolk extender. They also compared the effects of a one-step dilution, in which both diluents were added before equilibration, with those of a two-step dilution, in which the second diluent was added after equilibration and immediately before freezing. They also compared the effects of two freezing procedures, placing the straws horizontally above the LN₂ surface in a Styrofoam box (box method) or gradually lowering them vertically into a LN₂ tank (tank method). Finally, they compared the effects of warming the semen in a water bath at 70°C for 8 sec or warming at 37°C for 15 sec, on post-thaw survival of dog spermatozoa. The best treatment was to use Equex in the

diluent, to dilute the sperm in two steps, to freeze with the “box” method, and to warm in a water bath at 70°C for 8 sec.

Peña and Linde-Forsberg (2000b) also studied the effects on the post-thaw sperm motility and membrane integrity of (1) freezing dog semen at four sperm cell concentrations and (2) diluting the sperm immediately after thawing with Tris buffer at four concentrations. The results suggested that spermatozoa frozen in extender at concentrations of 200×10^6 sperm cells/ml and diluted 1:4 or 1:2 with a plain Tris buffer immediately post-thaw survived better than when frozen at lower or higher sperm concentrations and/or not diluted after thawing.

Nizanski et al. (2001) evaluated the post-thaw quality of ejaculated canine spermatozoa extended in two Tris-buffered diluents, either with or without detergent, and frozen in 0.25 ml or 0.5 ml straws or as pellets. Motility and acrosomal integrity of spermatozoa were significantly higher in semen samples frozen in pellets and in 0.5 ml straws compared to samples frozen in 0.25 ml straws. After thawing, there were no significant differences between samples frozen as pellets or in 0.5 ml French straws. Longevity of sperm motility was higher in samples diluted in Tris-buffered diluent with addition of Orvus ES Paste, regardless of the cryopreservation method.

Hewitt et al. (2001) derived a method for cryopreserving canine epididymal spermatozoa by adapting methods that had already been shown to be successful for ejaculated sperm. Epididymal spermatozoa were diluted with a Tris-based extender containing 2, 4, 6, or 8% glycerol and then were frozen in either 0.25 or 0.5 ml straws. Using the method that the authors found to be acceptable for

freezing canine epididymal sperm (6% glycerol, frozen in 0.5 ml straws), a sperm-oocyte penetration assay was then performed. There was no significant difference between the proportion of oocytes penetrated or the number of sperm penetrated per oocyte between the fresh and frozen-thawed sperm. The study showed that a protocol similar to that used for ejaculated sperm was suitable for the freezing of epididymal canine sperm.

Yu et al. (2002) studied the effect of cooling and warming rates on canine epididymal spermatozoa. The spermatozoa were diluted in DIMI medium containing 0.6 M glycerol in straws. With rapid thawing, motility was highest at a cooling rate of 11°C/min. With slow thawing, motility was highest at cooling rates of 3 to 11°C/min.

Assays of Survival of Spermatozoa

There are numerous assays, both *in vitro* and *in vivo*, that have been used to evaluate the survival of cryopreserved spermatozoa. “*In vitro*” refers to a process occurring in an artificial environment, such as a test tube or culture medium, while “*in vivo*” refers to a process occurring within a living body. *In vitro* assays of spermatozoa include determination of motility, membrane integrity, acrosome integrity, morphology, hypoosmotic swelling, zona-binding, *in vitro* fertilization (IVF), and intracytoplasmic sperm injection (ICSI). The most widely used *in vivo* assay of sperm survival is artificial insemination (AI).

Motility is an important functional characteristic of spermatozoa and is a widely used criterion to assess the effects of freezing spermatozoa of various species, including canids (England, 1993). Motility is routinely assessed by

subjective visual estimation of the percentage of motile cells (den Daas, 1992). Computer-assisted motility analysis methods have also been established for canids (Gunzel-Apel et al., 1993; Iguer-ouada and Versteegen, 2001a,b).

Membrane integrity refers to the intactness of the spermatozoon cellular membrane and is essential for a spermatozoon's ability to fertilize an oocyte on its own. Membrane integrity is routinely assessed by various staining techniques and also by the hypo-osmotic swelling test (De Leeuw et al., 1991; Harrison and Vickers, 1990; Merkies et al., 2000; Pintado et al., 2000; Thomas et al., 1997, 1998; Way et al., 1995). Eosin and nigrosin can be used as a differential stain to determine membrane integrity. In practice, eosinophilic and noneosinophilic cells are distinguished from each other, with a secondary stain (nigrosin) providing a background (Dott and Foster, 1972).

Garner and Johnson (1995) assessed the membrane integrity of spermatozoa from six mammalian species by a dual staining technique using the stains SYBR-14 and propidium iodide (PI). The former dye, SYBR-14, is a fluorescent nucleic acid stain that absorbs energy at 488 nm and emits it at 518 nm when bound to DNA. Microscopic examination revealed that SYBR-14 stained the nuclei of membrane-intact sperm bright green as determined by simultaneous examination of fluorescence and motility. Nonmotile sperm that had lost their membrane integrity were dyed bright red by PI. For individual damaged cells as their membranes became "leaky", staining with SYBR-14 was gradually replaced by staining with PI. Spermatozoa from bulls, boars, rams, rabbits, mice, and men were stained and examined through the use of

fluorescent microscopy. Similar staining patterns were observed in all six species tested. The fluorescent stains, SYBR-14 and propidium iodide, have since been used together to determine the proportion of membrane intact spermatozoa in a given sample of various mammalian species, including canids (Yu et al., 2002). Flow cytometry has been used in conjunction with these and other fluorescent stains to successfully determine membrane integrity (Peña et al., 1998a, 1999, 2001).

The hypo-osmotic swelling test has also been used to assess sperm membrane integrity (Smikle and Turek, 1997; Verheyen et al., 1997). Spermatozoa with intact membranes show swelling of the tail due to water influx when exposed to hypotonic conditions. This assay can be used to distinguish membrane-intact from membrane-damaged spermatozoa for ICSI (Verheyen et al., 1997). England and Plummer (1993) utilized a hypotonic swelling test to assess the membrane integrity of dog spermatozoa. The spermatozoa were evaluated for their ability to swell when exposed to hypotonic conditions. A medium in which both the number of swollen spermatozoa and their morphological appearance were influenced was developed to allow spermatozoal swelling to be identified easily. The number of swollen sperm was inversely proportional to the number of membrane-damaged sperm in a sample. Although spermatozoal swelling was highly repeatable, there was no correlation with sperm motility, morphology, or vital staining. Spermatozoa with damaged membranes were unable to swell. England and Plummer (1993) determined that

hypo-osmotic swelling of dog spermatozoa was reliable and repeatable and may be a useful assay for sperm membrane integrity.

The acrosome reaction of spermatozoa is an exocytotic event involving localized fusions between the outer acrosomal and sperm plasma membranes that results in formation of vesicles. The enzymes released during the acrosome reaction facilitate cumulus cell and zona penetration (Bedford, 1974). Because the acrosome reaction is necessary for fertilization, acrosome integrity is regarded as an important characteristic of sperm cells (den Daas, 1992) and has been assessed by various staining techniques (Cross and Meizel, 1989; De Leeuw et al., 1991; Maxwell and Johnson, 1997; Thomas et al., 1997; Way et al., 1995). Goodrowe et al. (2001) utilized Spermac® and fluorescein isothiocyanate-labelled *Pisum sativum* agglutinin lectin (PSA/FITC) stains to assess acrosome integrity of canine spermatozoa. Staining with Spermac®, a commercial proprietary product, allows the assessment of acrosomal integrity, and PSA/FITC staining allows assessment of acrosomal contents and integrity. Hewitt and England (1998, 1999) utilized Hoechst-33258 and chlortetracycline (CTC) to assess the acrosomes of dog sperm. The use of the fluorescent antibiotic, CTC, for the assessment of spermatozoal functional status has been shown to be particularly useful because, in addition to the determination of the acrosomal status of the spermatozoa, it allows for the further discrimination of acrosome intact cells between capacitated and uncapacitated groups. By co-staining with Hoechst-33258, the assessment of cell membrane integrity can be performed prior to CTC analysis, avoiding the assessment of membrane-

damaged spermatozoa as acrosome-reacted cells. Didion et al. (1989) utilized the Giemsa stain to determine acrosomal status. The acrosome stains light purple/dark pink while acrosome-free sperm remain unstained, which enables differentiation of spermatozoa that have undergone a true acrosome reaction from those which have undergone a false reaction.

Morphological defects of spermatozoa may be predictors of fertility. Sperm morphology can be analyzed using light or phase contrast microscopy, with or without staining (Mortimer, 1994; Oettle, 1993). Root-Kustritz et al. (1998) studied the types and the percentages of morphological abnormalities found in canine spermatozoa using three stains (Giemsa-Wright, eosin Y/nigrosin, and eosin B/nigrosin) with conventional light microscopy, compared with phase contrast microscopy on unstained samples. The percentage of spermatozoa with abnormal heads, midpieces, and tails varied by technique, but overall percentages of morphologically normal spermatozoa were consistent among assessment techniques.

Zona-binding and oocyte penetration assays can also be used to estimate whether stored germplasm of various species, including canids, has acceptable fertilizing potential (Gadea and Matas, 2000; Gadea et al., 1998; Goodrowe et al., 2001; Ivanova et al., 1999; Mastro Monaco et al., 2002). Mahi and Yanagimachi (1978) described a defined medium called “canine capacitation medium” (CCM) in which washed ejaculated canine spermatozoa could be induced to undergo capacitation and the acrosome reaction and to penetrate oocytes in vitro. When CCM was compared with two other commonly used

media, it allowed the best combination of good rates for acrosome reaction, motility, and zona penetration. Sperm-oocyte fusion also occurred when sperm and oocytes were incubated in CCM.

Hay et al. (1997b) studied the effects of cryopreservation on penetration of canine oocytes by spermatozoa. Slow cooling to 0°C did not adversely affect the ability of canine spermatozoa to penetrate oocytes. However, the addition of glycerol had a deleterious effect on gamete interaction despite little apparent change in morphology, membrane integrity, or acrosomal structure. Although sperm cells seemed to survive freezing, as determined by their membrane integrity and motility, there was a marked decrease in the number of spermatozoa capable of penetrating homologous oocytes.

Another study from the laboratory of Goodrowe (Hay et al., 1997a) showed that decreased motility and increased acrosomal damage to canine sperm cells was correlated with reduced penetration of homologous oocytes after freezing-thawing. That study also demonstrated that oocytes obtained from ovaries, either on the day of surgery or kept overnight, might be used to evaluate sperm cell penetrating potential.

Hewitt and England (1997) examined the canine oocyte penetration assay and its use as an indicator of the fertilizing potential of dog spermatozoa in vitro. The purpose of their study was to investigate the ability of in vitro capacitated canine spermatozoa to penetrate the zona pellucida of in vitro matured canine oocytes. No correlation was found between the acrosomal status of spermatozoa and spermatozoal penetration of homologous oocytes. In addition,

Hewitt and England found that the stage of oocyte nuclear maturation had no effect upon spermatozoal penetration and that immature oocytes could be penetrated by spermatozoa in this species.

Ivanova et al. (1999) used a hemi-zona assay to evaluate the capacity of fresh and frozen-thawed canine spermatozoa to bind to the zona pellucida. The hemizona assay provides a control for zona variability by allowing the comparison of spermatozoa treatments using matching hemizonae. The results of their study revealed that semen samples that exhibited similar characteristics before being frozen displayed different sperm-zona binding capacity after being thawed. Their data indicate that sperm-zona binding capacity after freezing is probably due to different freezing susceptibilities of spermatozoa from individual dogs.

In vitro fertilization (IVF) is another method of evaluating the fertilizing ability of spermatozoa in many mammalian species (Blash et al., 2000; Commizoli et al., 2001; Graff et al., 1996; Nagai et al., 1988; Rath and Niemann, 1997; Songsasen et al., 1997, 1998). Due to the unique physiology of the canine female, IVF has not been successful in canids (Farstad, 2000b), and very few canine embryos have been produced by IVF (Otoi et al., 2000; Yamada et al., 1992, 1993). However, in a recent study, England et al. (2001) reported a pregnancy following in vitro fertilization of canine oocytes recovered from ovaries at standard ovariohysterectomies. After insemination, culture, and embryo transfer, a pregnancy was obtained but no birth was reported. The study of England et al. (2001) was the first to document that in vitro fertilization of oocytes

and subsequent implantation of resultant embryos can be achieved in canines. Williams et al. (2001) have produced canine embryos in vitro by fertilization with both cooled and cryopreserved epididymal spermatozoa. Numerous pregnancies and live births have been reported from transfer of IVF-produced embryos of various mammalian species (Graff et al., 1996; Songsasen et al., 1997, 1998), but transfer of canine embryos has been much less successful.

ICSI is another useful technique to assess sperm fertilizing ability and to produce embryos in vitro of some species (Cayan et al., 2001; McLachlan, 1998). Since epididymal sperm exhibits reduced fertilization and lower pregnancy rates when used for IVF, ICSI may be especially useful for embryo production with epididymal spermatozoa (McLachlan, 1998). Fulton et al. (1998) performed ICSI using canine gametes and observed the formation of pronuclei in the sperm-injected ova.

AI is considered to be an in vivo assay of spermatozoa survival and is the most definitive test of sperm function. Artificial insemination can be used to determine fertility in vivo and has been widely and successfully used in many mammalian species (Chandler, 2000; Januskauskas et al., 1999; Nakatsukasa et al., 2001; Pursel et al., 1972; Pursel and Johnson, 1975; Vicente and Viudes-de-Castro, 1996). Artificial insemination with fresh and frozen-thawed ejaculated spermatozoa has been used widely and successfully in canines (Farstad, 1984; Farstad and Andersen Berg, 1989; Linde-Forsberg and Forsberg, 1989, 1993; Nöthling, 1995; Seager et al., 1975; Thomassen et al., 2001).

Epididymal spermatozoa have not been as widely used for AI as ejaculated spermatozoa. In a study using AI to determine the fertility of bovine spermatozoa, Amann and Griel (1973) reported that the fertilizing capacities of cauda epididymal and ejaculated spermatozoa were not significantly different. Epididymal canine spermatozoa have been shown to have the ability to fertilize oocytes in vivo (Marks et al., 1994).

CHAPTER 3

MATERIALS AND METHODS

Overall Experimental Design

Four separate experiments were conducted in this investigation. These consisted of the following:

- I. Effect of pellet volumes on post-thaw survival of epididymal spermatozoa from individual dogs
- II. Effect of CPAs and thawing solutions on post-thaw survival of epididymal spermatozoa from individual dogs
- III. Capacity of epididymal spermatozoa from individual dogs to bind to canine zonae prior to pellet-freezing and after thawing
- IV. Pellet freezing of ejaculated spermatozoa from individual dogs

General Experimental Methods

Preparation of Testes

In four series of experiments, testes were obtained from a total of 50 dogs undergoing standard orchiectomy at local veterinary clinics. Immediately after surgical removal, testes with epididymides were placed in plastic specimen bags containing sterile saline supplemented with penicillin at 100 IU/ml and streptomycin at 0.1 mg/ml. All chemicals were obtained from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated. Specimens were stored and transported to the laboratory at ambient temperature (15 to 25°C). The time between surgery and the collection of spermatozoa was as short as 3 hr, but sometimes was as long as 20 hr.

Collection of Epididymal Spermatozoa

Canine epididymal spermatozoa were collected for cryopreservation and processed using the methods described by Yu et al. (2002). When the specimens arrived at the laboratory, caudal epididymides were dissected free from the testes of each dog in a disposable 100 mm Petri dish (Falcon Plastics, Franklin Lakes, NJ, USA), rinsed with 0.9% saline solution, and placed into a disposable 35 mm Petri dish (Falcon Plastics) containing 2 ml of modified Tyrode's salt solution (TALP: Tyrode's solution plus albumin, lactate, pyruvate), as described by Parrish et al. (1988). Epididymides were cut repeatedly with sterile surgical scissors, and spermatozoa were allowed to swim out into the solution for 10 min at 37°C in a humidified atmosphere of 5% CO₂ in 95% air. Each suspension of spermatozoa was filtered through a cell strainer (Model 2840; Falcon Plastics) to remove tissue debris, and the filtrate was transferred into a sterile 15 ml plastic centrifuge tube (Falcon Plastics) with a sterile pipet.

Percoll Gradient Centrifugation of Motile, Membrane-Intact Spermatozoa

Percoll solutions and gradients were prepared as described by Chan et al. (1994), Guerin et al. (1989), and Mortimer (1994). A 100% Percoll stock solution was prepared by mixing nine volumes of Percoll (Sigma Chemical Company, St. Louis, MO, USA) with one volume of 10x TALP solution. Two Percoll solutions of 90% and 45% (v/v) were prepared by diluting 100% stock Percoll solution with 1x TALP. A two-layer discontinuous gradient was obtained by layering 1.5 ml of 45% Percoll solution on top of 1.5 ml of 90% Percoll solution in a 15 ml sterile centrifuge tube. Up to 1.5 ml of epididymal sperm suspension was layered on

top of each two-layer discontinuous Percoll gradient and centrifuged for 20 min at 300 × g at room temperature. After removal of the supernatant, the pellet was washed with 4 ml of TALP by centrifugation at 300 × g for 5 min (Centra Model CL2 Centrifuge, International Equipment Company, Needham Heights, MA, USA). The supernatant was removed, and the pellet was held at room temperature (22 to 25°C) until used.

Preparation of Diluents

The first diluent was DIMI medium, and the second diluent was DIMI + 0.87 M CPA (Foote, 1964). The composition of the DIMI medium was 0.153 M lactose, 0.034 M sodium citrate, 0.063 M glycine, 0.010 M sulfanilamide, and 0.009 M sodium dodecyl sulfate (SDS) supplemented with 100 IU/ml penicillin, 0.1 mg/ml streptomycin sulfate, and 20% egg yolk (EY). The DIMI medium was modified by the addition of lactose in place of arabinose and glucose because lactose has been shown to produce higher post-thaw survival for pellet-frozen dog spermatozoa compared with other sugars in extenders used for pellet-freezing (Seager, 1969; Seager and Fletcher, 1973). The egg yolk solution was prepared as described by Songsasen and Leibo (1997) and was solubilized by the addition of 0.25% SDS into the medium. The mean osmolality of the solution, as measured by a calibrated Osmette osmometer, was 400 ± 5 mOsm with a pH of 6.9.

Dilution of Spermatozoa Samples

The cell concentration of the washed sperm pellet was determined using a Bright-Line Hemocytometer (Hausser Scientific, Horsham, PA, USA). After being

counted, the cell concentration was adjusted to 20×10^6 cells/ml by dilution with DIMI medium. The diluted spermatozoa were held at room temperature (22 to 25°C) for 10 min, and the percentages of motile and membrane-intact spermatozoa of each specimen were assessed in triplicate. Sperm suspensions with $\geq 60\%$ motility and $\geq 75\%$ membrane integrity were utilized for further study. Aliquants of the sperm suspension were then further diluted with equal volumes of DIMI containing 0.87 M CPA to achieve final concentrations of 0.43 M CPA and 10×10^6 cells/ml. In these experiments, a final CPA concentration of 0.43 M, which is $\sim 4\%$ (v/v), was used. Seager (1969) indicated that 4% (v/v) was the glycerol concentration that resulted in the overall maximum survival of spermatozoa after thawing. A glycerol concentration of approximately 4% (v/v) also resulted in optimal survival in several other studies of the freezing of canine epididymal spermatozoa (Fontbonne and Badinand, 1993; Hewitt et al., 2001; Olar et al., 1989; Rota et al., 1998).

Procedures for Pellet Freezing Spermatozoa

All aliquants were then divided into three portions. One portion of each aliquant was held at room temperature (22 to 25°C), and a second portion was cooled to 4°C at about $-0.2^\circ\text{C}/\text{min}$ by placing it into a standard refrigerator for two hours and was held at that temperature to serve as a control for the cryopreserved samples. The third portion of the final sperm suspension to be cryopreserved was also cooled to 4°C at about $-0.2^\circ\text{C}/\text{min}$. Cooled samples from the third portion were frozen as pellets on a block of dry ice (Insta-Ice™ Dry Ice Machine, Model 460; Polyfoam Packers Corp., Wheeling, IL, USA) with

indentations on its surface. The weights of the pellet volumes were determined by weighing the appropriate volume of medium measured with an Eppendorf pipettor. To freeze the sperm in pellet form, indentations were made on the surface of the dry ice by placing a 96-well culture dish on the inside wall of the Insta-Ice™ Dry Ice Machine. Cooled sperm suspensions were delivered to the indentations on the dry ice by Eppendorf pipettors set to the volumes desired, and the suspensions were frozen in the form of pellets (Figure 3.1). Once frozen on the dry ice, sperm pellets were plunged and stored in plastic ampules in LN₂ for at least 30 min. Pellets were removed from the plastic ampules by forceps under LN₂, and each pellet was dropped immediately upon removal from the LN₂ into a 1 ml plastic Eppendorf tube in a 37°C water bath containing the appropriate volume of solution for a 1:3 dilution while being swirled vigorously for 30 sec.

Assessment of Survival of Spermatozoa

All fresh, cooled/pre-freeze, unfrozen, and frozen-thawed samples were analyzed to determine their survival based on assays of motility and membrane integrity. The percentage of progressively motile spermatozoa was assessed subjectively by microscopic examination at 200 × magnification of specimens warmed and evaluated at 37°C on a transparent slide warmer. For each replicate of each sample, three microscope fields were examined to estimate percentage motility. The integrity of sperm plasma membranes was measured using the Live/Dead Sperm Viability Kit (Molecular Probes, Eugene, OR, USA) containing the fluorescent stains SYBR-14 and propidium iodide.

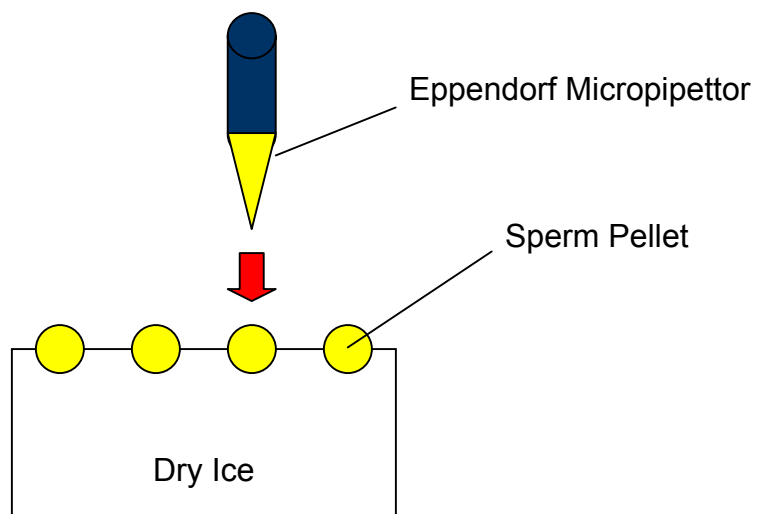


Figure 3.1. Pellet freezing of spermatozoa on dry ice. To freeze the sperm in pellet form, indentations were made on the surface of the dry ice by placing a 96-well culture dish on the inside wall of the Insta-Ice™ Dry Ice Machine. Cooled sperm suspensions were delivered to the indentations on the dry ice by an Eppendorf micropipettor set to the volumes desired, and the suspensions were frozen in the form of pellets.

For each replicate of each sample, at least 200 spermatozoa were counted in several microscope fields. With this assay, spermatozoa that exhibit green fluorescence are considered membrane-intact and those that exhibit red fluorescence are considered to have damaged membranes (Figure 3.2). The percentage of membrane-intact spermatozoa was calculated as a fraction of the total.

Oocyte Collection and Freezing

Canine oocytes were collected and cryopreserved for use in the zona-binding assay using the method described by Yu and Leibo (2002). Canine ovaries were obtained from bitches undergoing ovariohysterectomy at local veterinary clinics. Ovaries were transported in plastic bags containing sterile 0.9% saline solution at ambient temperature (15 to 25°C). Ovaries were rinsed with 0.9% saline, placed in a Petri dish containing Dulbecco's PBS (D-PBS), and the tissue was minced into small pieces with a sterile scalpel blade to release cumulus-oocyte-complexes. Oocytes were considered of good quality if they were surrounded by at least 3 to 4 layers of cumulus cells and had homogeneous cytoplasm. The oocytes determined to be of good quality were transferred to 3 ml of 3% sodium citrate buffer in a 5 ml test tube and shaken vigorously to remove cumulus cells. The oocytes were then washed in D-PBS containing 0.4% bovine serum albumin and placed into straws. The straws were heat-sealed, placed in a -20°C freezer overnight, and then plunged into liquid nitrogen for storage.

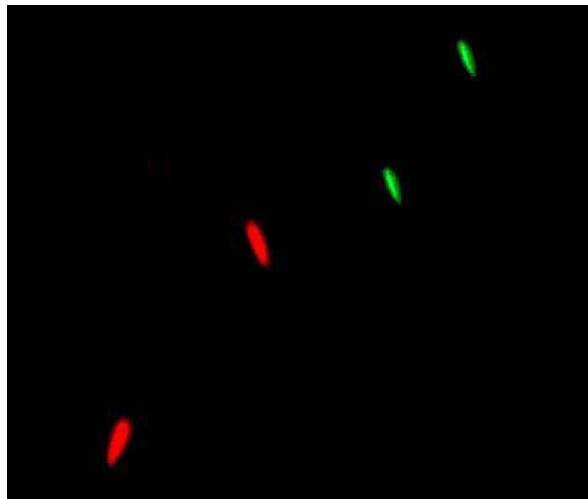


Figure 3.2. Representative membrane integrity assay. Spermatozoa that exhibit green fluorescence due to SYBR-14 staining are considered membrane-intact, and those that exhibit red fluorescence due to propidium iodide staining are considered to have damaged membranes.

Zona-Binding Assay

For the zona-binding assay, oocytes frozen in straws were thawed for 1 min at 37°C, washed in CCM (Kim et al., 1995), and were incubated in CCM for 10 to 20 min in a humidified atmosphere of 5% CO₂ in air at 37°C. Unfrozen and frozen-thawed spermatozoa were diluted with CCM to achieve a concentration of 5×10^6 spermatozoa/ml. Sperm droplets of 100 µl each were made under 1 ml of sterile mineral oil. Canine oocytes were placed in the sperm drops and were incubated under oil for 1 min. The oocytes were then washed to remove loosely bound sperm by pipetting them vigorously in fresh CCM and were incubated for 1 hr in a droplet of fresh CCM under oil at 38.5°C in a humidified atmosphere of 5% CO₂ in 95% air. Oocytes and sperm were then stained with the fluorescent labels SYBR-14 and propidium iodide (Live/Dead Sperm Viability Kit, Molecular Probes, Eugene, OR, USA) and viewed using fluorescent microscopy to detect the presence of membrane-intact spermatozoa bound to the zonae (Figure 3.3) (Yu et al., 2002).

Experiment I: Effect of Pellet Volumes on Post-Thaw Survival of Epididymal Spermatozoa from Individual Dogs

Experimental Design

For sperm samples collected from each of 15 dogs, duplicate sets of pellets were frozen at each pellet volume and stored in LN₂ while a small volume of the same suspension was held at 4°C to serve as a pre-freeze control. After thawing, each pellet sample was assessed in triplicate to determine percentages of motility and membrane integrity.

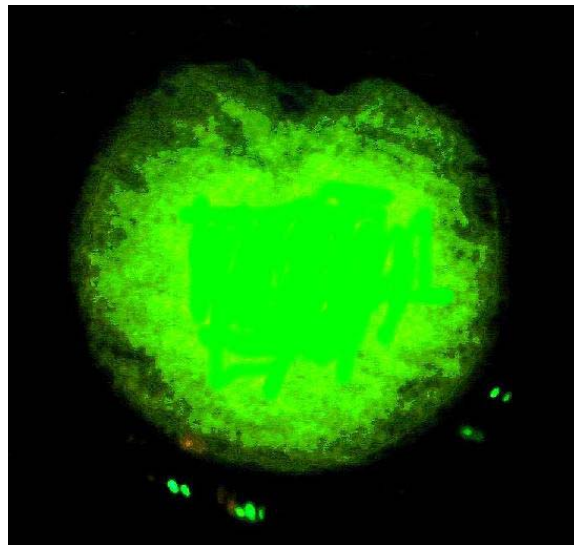
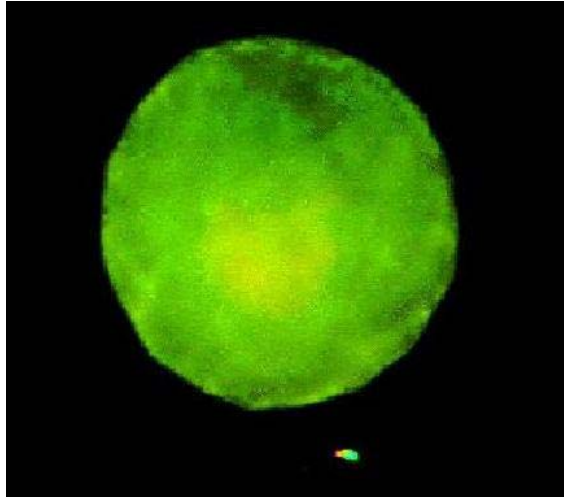


Figure 3.3. Representative zona-binding assays. Oocytes and sperm were stained with the fluorescent labels SYBR-14 and propidium iodide and viewed using a fluorescent microscope (40 x magnification) to detect the presence of membrane-intact spermatozoa bound to the zona pellucida. The large fluorescent circles are the nuclear and cytoplasmic material of the oocyte, and the small green fluorescent spots are membrane-intact spermatozoa bound to the zona pellucida of each oocyte.

Collection and Preparation of Testes

Testes were obtained from 15 dogs undergoing standard orchiectomy at local veterinary clinics during the period from July 16, 2002 to August 8, 2002. The testes that were used were obtained from privately owned dogs of pure and mixed breeds, ranging in age from 8 months to 6 years. The identified breeds were: Blue Heeler, Boxer, Golden Retriever, Labrador Retriever, Rottweiler, West Highland White Terrier, and Yorkshire Terrier. The breeds of some dogs were not identified at the time of orchiectomy. Testes were prepared as described above.

Procedures for Pellet-Freezing Spermatozoa

Cooled samples were frozen as 10, 50, 100, or 200 μ l pellets on dry ice. The respective average pellet weights of 10 pellets of each volume were 0.0103 ± 0.0006 g for 10 μ l, 0.0504 ± 0.0007 g for 50 μ l, 0.1043 ± 0.0024 g for 100 μ l, and 0.2041 ± 0.0014 g for 200 μ l. Once frozen on the dry ice, sperm pellets were plunged and stored in plastic ampules in LN₂ for at least 30 min. Samples were thawed by a 1:3 dilution in TALP, each pellet being dropped into a 1 ml plastic Eppendorf tube containing the appropriate volume of TALP for the correct dilution, in a 37°C water bath while being swirled vigorously for 30 sec.

Collection, Preparation, and Analysis of Spermatozoa

Collection of epididymal spermatozoa and Percoll gradient centrifugation of motile, membrane-intact sperm were performed as described in General Experimental Methods. Aliquants of the sperm suspension were diluted to achieve final concentrations of 10×10^6 cells/ml suspended in 0.43 M glycerol in

DIMI. Spermatozoa survival was determined by microscopic evaluation of motility and membrane integrity as described in General Experimental Methods.

Experiment II: Effect of CPAs and Thawing Solutions on Post-Thaw Survival of Epididymal Spermatozoa from Individual Dogs

Experimental Design

For sperm samples collected from each of 16 dogs, triplicate sets of 100 μ l pellets were frozen for each CPA and stored in LN₂ while small volumes were held at 4°C to serve as a pre-freeze controls for each CPA used. One pellet from each CPA treatment was thawed in each thawing solution. Each post-thaw pellet sample was assessed in triplicate to determine percentages of motility and membrane integrity.

Preparation of Testes

For this experiment, testes were obtained from 16 dogs undergoing standard orchiectomy at local veterinary clinics during the period from August 12, 2002 to August 23, 2002. The testes used were obtained from privately owned dogs of pure and mixed breeds, ranging in age from 6 months to 6 years. The identified breeds of dogs used in this experiment were: Dachshund, Dalmatian, Golden Retriever, Papillon, Pit Bull, Pomeranian, Poodle, Rottweiler, and Siberian Husky. The breeds of some dogs were not identified. Testes were prepared as described in the General Experimental Methods.

Procedures for Pellet Freezing Spermatozoa

All of the samples in this experiment were frozen on dry ice as 100 μ l pellets, the pellet volume found in Experiment I to produce the highest survival as determined by motility and membrane integrity analysis. The average weights of

10 pellets each of glycerol, DMSO, and EG diluents were: 0.1043 ± 0.0024 g, 0.0997 ± 0.0008 g, and 0.0984 ± 0.0008 g, respectively. The frozen pellets were plunged and stored in LN₂ for at least 30 min. Samples containing each CPA were thawed by 1:3 dilution in either TALP, Canine Capacitation Medium (CCM), as described by Mahi and Yanagimachi (1978), or in 3% sodium citrate solution in a 37°C water bath while being swirled vigorously for 30 sec.

Collection, Preparation, and Analysis of Spermatozoa

Collection of epididymal spermatozoa and Percoll gradient centrifugation of motile, membrane-intact sperm were performed as described in General Experimental Methods. Sperm samples were diluted to a final concentration of 10×10^6 cells/ml with DIMI containing glycerol, ethylene glycol, or dimethyl sulfoxide to achieve final CPA concentrations of 0.43 M. Spermatozoa survival was determined by microscopic evaluation of motility and membrane integrity as described in General Experimental Methods.

Experiment III: Capacity of Epididymal Spermatozoa from Individual Dogs to Bind to Canine Zona Prior to Pellet Freezing and after Thawing

Experimental Design

For sperm collected from each of 15 dogs, duplicate samples of spermatozoa were frozen as pellets and stored in LN₂ while a portion was held at room temperature to serve as an unfrozen control. Each pellet was used to prepare two sperm droplets, each of which contained six oocytes, to be used for the zona-binding assay. Unfrozen spermatozoa were also used in duplicate sets of two sperm droplets, each containing six oocytes, to serve as a control. Each post-thaw pellet sample was assessed in triplicate to determine percentages of

motility and membrane integrity and was also assessed for zona-binding capacity.

Preparation of Testes

For this experiment, testes were obtained from 15 dogs undergoing standard orchiectomy at local veterinary clinics during the period from September 4, 2002 to September 23, 2002. The testes used were obtained from privately owned dogs of pure and mixed breeds, ranging in age from 1 year to 8 years. The identified breeds were Beagle, Bichon Frise, Boxer, Chihuahua, Chow Chow, Cocker Spaniel, German Pointer, German Shepard, Laborador Retriever, Pit Bull, and Samoyed, while the breeds of some dogs were not identified in this experiment. Testes were prepared as described previously.

Procedures for Pellet Freezing Spermatozoa

Cooled sperm samples were frozen as 100 μ l pellets on Dry Ice. Samples were thawed by a 1:3 dilution in CCM, the thawing solution found in Experiment II to produce the highest survival, in a 37°C water bath while being swirled vigorously for 30 sec.

Collection, Preparation, and Analysis of Spermatozoa and Oocytes

Collection of epididymal spermatozoa and Percoll gradient centrifugation of motile, membrane-intact sperm were performed as described in General Experimental Methods. Sperm samples were diluted to a final concentration of 10×10^6 cells/ml with DIMI containing glycerol, the CPA found in Experiment II to produce the highest survival. The final cryoprotective agent concentrations were 0.43 M for the portion to be cryopreserved, or 0 M CPA for the unfrozen portion.

Spermatozoa survival was determined by microscopic evaluation of motility and membrane integrity as well as by the zona-binding assay described in General Experimental Methods.

Canine oocytes were collected and prepared for use in the zona-binding assay using the method described in General Experimental Methods.

Experiment IV: Pellet Freezing of Ejaculated Spermatozoa from Individual Dogs

Experimental Design

Having previously used epididymal sperm to determine the influence of several variables on canine spermatozoa survival, ejaculated spermatozoa from three dogs were used to determine the reproducibility of the post-thaw survival of spermatozoa within an individual dog. For each of the three ejaculates from each of three dogs and for sperm collected from the epididymides of each of four dogs, duplicate sets of spermatozoa were frozen as pellets and stored in LN₂ while a portion was held at room temperature to serve as an unfrozen control.

Each pellet was used to prepare two sperm droplets, each of which contained six oocytes, used for the zona-binding assay. Unfrozen spermatozoa were also used in duplicate sets of two sperm droplets, each containing six oocytes, to serve as a control. Each post-thaw pellet sample was assessed in triplicate to determine percentages of motility and membrane integrity and was also assessed for zona-binding capacity. For ejaculated samples from each dog, there were three ejaculates and two pellets/ejaculate, and each pellet was assayed in triplicate, for a total of 18 assays/dog.

Collection and Preparation of Ejaculated Spermatozoa

On separate occasions, triplicate ejaculates were obtained from each of three mixed-breed hounds of proven fertility housed at the Louisiana State University School of Veterinary Medicine (LSU SVM). For each collection, ejaculates were evaluated for volume, concentration, and progressive motility and were diluted 1:1 with Kenney skim milk extender (Lane Manufacturing, Inc., Denver, CO, USA). The diluted ejaculates were then transported from the LSU SVM in Baton Rouge, LA to our laboratory in New Orleans, LA (~2 hr). The time between collection and use was ~3 hr. Upon arrival at our laboratory, the diluted ejaculates were centrifuged at $300 \times g$ for 5 min. The sperm pellets were then centrifuged on a Percoll gradient as described in General Experimental Methods.

Preparation of Testes

For this experiment, testes were obtained from four dogs undergoing standard orchiectomy at local veterinary clinics during the period from November 5, 2002 to November 14, 2002. The testes used were obtained from privately owned dogs of pure and mixed breeds, ranging in age from 8 months to 2 years. The identified breeds were Chihuahua and Cocker Spaniel. The breeds of some dogs were not identified. Testes were prepared for this study as described above.

Procedures for Pellet Freezing Spermatozoa

The unfrozen portion of diluted spermatozoa containing no glycerol was held at room temperature (22 to 25°C) while the cryopreserved portion was being cooled, frozen, and thawed. The portion containing 0.43 M glycerol was cooled

to 4°C. Cooled sperm samples were frozen as 100 µl pellets on dry ice. The frozen pellets were plunged and stored in LN₂. While being swirled vigorously for 30 sec, samples were thawed in a 37°C water bath by 1:3 dilution in CCM.

Collection, Preparation, and Analysis of Spermatozoa and Oocytes

Collection of epididymal spermatozoa and Percoll gradient centrifugation of motile, membrane-intact sperm were performed as described in General Experimental Methods. Sperm samples were diluted to a final concentration of 10×10^6 cells/ml with DIMI containing 0.87 M glycerol, the CPA found in Experiment II to produce the highest survival, to achieve a final concentration of 0.43 M for the portion to be cryopreserved, or without any CPA for the unfrozen portion. Spermatozoa survival was determined by microscopic evaluation of motility and membrane integrity as well as by the zona-binding assay described in General Experimental Methods.

Canine oocytes were collected and cryopreserved for use in the zona-binding assay as described in General Experimental Methods.

Statistical Analysis

Data on motility, membrane integrity, and membrane-intact sperm bound per oocyte were compared by analysis of variance using the Mixed Procedure (modified General Linear Means) of SAS Version 8, and the results were compared using Tukey's Studentized Range test (SAS, 1985). Factors that were included in the models were pellet volume, CPA, thawing solution, fresh, cooled/pre-freeze, unfrozen, or frozen-thawed status of samples, and individual

dogs. Correlations between motility, membrane integrity, and sperm bound to zona were analyzed by the SAS Linear Regression Procedure (SAS, 1985).

CHAPTER 4

RESULTS

Experiment I

In this experiment, the effect of pellet volumes on post-thaw survival of epididymal spermatozoa from individual dogs was determined. Spermatozoa were suspended in 0.43 M glycerol, frozen as 10, 50, 100, or 200 μ l pellets, and were thawed in TALP. The membrane integrity and motility of replicate samples of pre-freeze and frozen-thawed spermatozoa are shown in Tables 4.1 and 4.2. Tables 4.1.A and 4.1.B contain the same membrane integrity data, but, in Table 4.1.A, means are compared within each individual dog and, in Table 4.1.B, means are compared within each treatment group. Tables 4.2.A and 4.2.B contain the same motility data, but Table 4.2.A means are compared within each individual dog and Table 4.2.B means are compared within each treatment group. Fresh samples had significantly higher survival than the cooled and frozen-thawed samples, and the cooled (pre-freeze controls) samples had significantly higher survival than the frozen-thawed samples (Tables 4.1 and 4.2).

The post-thaw sperm survival was dependent on the pellet volume ($P < 0.05$), as shown by results in Tables 4.1 and 4.2. The highest mean post-thaw values of membrane integrity were obtained with pellet volumes of 100 μ l and 200 μ l (Figure 4.1). As also shown in Figure 4.1, the mean percentage of motile spermatozoa was low at a pellet volumes of 10 μ l and 50 μ l, increased to an apparent maximum with pellets of a 100 μ l volume, and slightly decreased at a pellet volume of 200 μ l.

Table 4.1. Effect of pellet volume on membrane integrity of epididymal spermatozoa.

A. Comparison of percentage membrane integrity within each individual dog.

Dog	Membrane Integrity (Mean % ± SEM)				
	Pre-freeze	10 µl	50 µl	100 µl	200 µl
1	90.0 ± 1.4 ^a	24.5 ± 1.4 ^{bc}	35.0 ± 1.9 ^b	20.7 ± 1.6 ^c	19.8 ± 1.5 ^c
2	90.8 ± 1.9 ^a	19.7 ± 2.2 ^d	38.8 ± 1.6 ^{bc}	45.5 ± 4.1 ^b	31.5 ± 2.1 ^{cd}
3	89.0 ± 2.0 ^a	22.5 ± 2.4 ^c	20.8 ± 1.5 ^c	36.8 ± 2.5 ^b	23.3 ± 1.8 ^c
4	92.7 ± 1.2 ^a	9.3 ± 1.6 ^d	29.8 ± 3.1 ^c	53.8 ± 2.0 ^b	36.5 ± 2.8 ^c
5	92.5 ± 1.4 ^a	2.3 ± 0.7 ^e	30.3 ± 2.9 ^d	45.2 ± 1.7 ^c	61.5 ± 3.0 ^b
6	87.2 ± 1.2 ^a	7.3 ± 1.9 ^d	31.5 ± 2.4 ^c	55.3 ± 3.7 ^b	40.2 ± 1.2 ^c
7	93.5 ± 1.9 ^a	0.5 ± 0.3 ^d	20.8 ± 1.7 ^c	29.5 ± 1.7 ^c	53.8 ± 2.7 ^b
8	78.3 ± 1.3 ^a	13.8 ± 1.4 ^c	17.8 ± 2.0 ^c	37.0 ± 2.9 ^b	38.0 ± 3.6 ^b
9	82.2 ± 2.4 ^b	10.5 ± 1.4 ^e	41.0 ± 2.7 ^d	72.0 ± 2.1 ^{bc}	61.2 ± 2.9 ^c
10	92.2 ± 2.4 ^a	2.8 ± 0.6 ^d	17.5 ± 1.4 ^c	31.7 ± 2.6 ^b	23.0 ± 1.6 ^{bc}
11	84.8 ± 1.7 ^a	2.7 ± 1.0 ^d	27.0 ± 1.5 ^c	33.0 ± 1.7 ^c	58.2 ± 2.0 ^b
12	79.3 ± 2.9 ^a	7.8 ± 2.1 ^d	18.2 ± 1.6 ^{cd}	32.2 ± 2.4 ^b	23.8 ± 1.7 ^{bc}
13	88.7 ± 0.9 ^a	17.7 ± 1.6 ^d	25.8 ± 2.4 ^d	65.7 ± 1.9 ^b	41.0 ± 1.5 ^c
14	86.3 ± 1.5 ^a	12.7 ± 1.9 ^d	25.3 ± 3.0 ^c	47.8 ± 2.7 ^b	22.0 ± 1.9 ^{cd}
15	86.2 ± 1.5 ^a	13.2 ± 1.6 ^c	33.0 ± 2.0 ^b	38.2 ± 2.8 ^b	34.5 ± 2.9 ^b
MEAN	87.6 ± 0.6 ^a	11.2 ± 0.9 ^d	27.5 ± 0.9 ^c	43.0 ± 1.6 ^b	37.9 ± 1.6 ^b

Values with different superscripts within a row are significantly different (P<0.05).

B. Comparison of percentage membrane integrity within each treatment group.

Dog	Membrane Integrity (Mean % ± SEM)				
	Pre-freeze	10 µl	50 µl	100 µl	200 µl
1	90.0 ± 1.4 ^{abc}	24.5 ± 1.4 ^a	35.0 ± 1.9 ^{abc}	20.7 ± 1.6 ^g	19.8 ± 1.5 ^d
2	90.8 ± 1.9 ^{ab}	19.7 ± 2.2 ^{abc}	38.8 ± 1.6 ^{ab}	45.5 ± 4.1 ^{cd}	31.5 ± 2.1 ^{bcd}
3	89.0 ± 2.0 ^{ab}	22.5 ± 2.4 ^{ab}	20.8 ± 1.5 ^{def}	36.8 ± 2.5 ^{def}	23.3 ± 1.8 ^{cd}
4	92.7 ± 1.2 ^a	9.3 ± 1.6 ^{cde}	29.8 ± 3.1 ^{abcdef}	53.8 ± 2.0 ^{bc}	36.5 ± 2.8 ^b
5	92.5 ± 1.4 ^a	2.3 ± 0.7 ^{de}	30.3 ± 2.9 ^{abcde}	45.2 ± 1.7 ^{cde}	61.5 ± 3.0 ^a
6	87.2 ± 1.2 ^{abc}	7.3 ± 1.9 ^{cde}	31.5 ± 2.4 ^{abcd}	55.3 ± 3.7 ^{bc}	40.2 ± 1.2 ^b
7	93.5 ± 1.9 ^a	0.5 ± 0.3 ^e	20.8 ± 1.7 ^{def}	29.5 ± 1.7 ^{fg}	53.8 ± 2.7 ^a
8	78.3 ± 1.3 ^c	13.8 ± 1.4 ^{abcd}	17.8 ± 2.0 ^f	37.0 ± 2.9 ^{def}	38.0 ± 3.6 ^b
9	82.2 ± 2.4 ^{abc}	10.5 ± 1.4 ^{bcde}	41.0 ± 2.7 ^a	72.0 ± 2.1 ^a	61.2 ± 2.9 ^a
10	92.2 ± 2.4 ^a	2.8 ± 0.6 ^{de}	17.5 ± 1.4 ^f	31.7 ± 2.6 ^{fg}	23.0 ± 1.6 ^{cd}
11	84.8 ± 1.7 ^{abc}	2.7 ± 1.0 ^{de}	27.0 ± 1.5 ^{bcdef}	33.0 ± 1.7 ^{efg}	58.2 ± 2.0 ^a
12	79.3 ± 2.9 ^{bc}	7.8 ± 2.1 ^{cde}	18.2 ± 1.6 ^{ef}	32.2 ± 2.4 ^{fg}	23.8 ± 1.7 ^{cd}
13	88.7 ± 0.9 ^{abc}	17.7 ± 1.6 ^{abc}	25.8 ± 2.4 ^{cdef}	65.7 ± 1.9 ^{ab}	41.0 ± 1.5 ^b
14	86.3 ± 1.5 ^{abc}	12.7 ± 1.9 ^{abcde}	25.3 ± 3.0 ^{cdef}	47.8 ± 2.7 ^{cd}	22.0 ± 1.9 ^d
15	86.2 ± 1.5 ^{abc}	13.2 ± 1.6 ^{abcd}	33.0 ± 2.0 ^{abcd}	38.2 ± 2.8 ^{def}	34.5 ± 2.9 ^{bc}

Values with different superscripts within a column are significantly different (P<0.05).

Table 4.2. Effect of pellet volume on motility of epididymal spermatozoa.
A. Comparison of percentage motility within each individual dog.

Dog	Motility (Mean % \pm SEM)				
	Pre-freeze	10 μ l	50 μ l	100 μ l	200 μ l
1	80.0 \pm 1.8 ^a	15.0 \pm 1.8 ^c	24.2 \pm 1.5 ^b	12.5 \pm 1.1 ^{cd}	6.7 \pm 2.1 ^d
2	82.5 \pm 1.1 ^a	4.2 \pm 1.5 ^d	13.3 \pm 1.7 ^c	33.3 \pm 1.7 ^b	10.8 \pm 1.5 ^{cd}
3	78.3 \pm 1.1 ^a	4.2 \pm 1.5 ^c	10.8 \pm 1.5 ^c	20.0 \pm 1.3 ^b	7.5 \pm 1.7 ^c
4	91.7 \pm 1.1 ^a	2.5 \pm 1.1 ^d	25.0 \pm 1.3 ^c	51.7 \pm 1.7 ^b	23.3 \pm 1.7 ^c
5	87.5 \pm 1.1 ^a	0.8 \pm 0.8 ^e	14.2 \pm 1.5 ^d	35.0 \pm 2.6 ^c	45.8 \pm 1.5 ^b
6	82.5 \pm 1.1 ^a	0.8 \pm 0.8 ^e	15.8 \pm 1.5 ^d	54.2 \pm 1.5 ^b	34.2 \pm 1.5 ^c
7	88.3 \pm 1.1 ^a	0.0 \pm 0.0 ^d	9.2 \pm 1.5 ^c	23.3 \pm 1.1 ^b	27.5 \pm 1.1 ^b
8	73.3 \pm 1.7 ^a	0.0 \pm 0.0 ^c	6.7 \pm 1.1 ^c	36.7 \pm 2.1 ^b	29.2 \pm 1.5 ^b
9	67.5 \pm 1.1 ^a	0.8 \pm 0.8 ^d	5.8 \pm 0.8 ^d	39.2 \pm 1.5 ^b	24.2 \pm 0.8 ^c
10	82.5 \pm 1.1 ^a	0.0 \pm 0.0 ^d	10.8 \pm 1.5 ^c	22.5 \pm 1.1 ^b	19.2 \pm 1.5 ^b
11	75.8 \pm 1.5 ^a	0.8 \pm 0.8 ^d	18.3 \pm 1.1 ^c	19.2 \pm 0.8 ^c	44.2 \pm 1.5 ^b
12	72.5 \pm 1.1 ^a	4.2 \pm 0.8 ^d	9.2 \pm 1.5 ^{cd}	29.2 \pm 0.8 ^b	16.2 \pm 1.7 ^c
13	85.0 \pm 1.3 ^a	15.0 \pm 1.3 ^d	15.8 \pm 0.8 ^{cd}	49.2 \pm 1.5 ^b	23.3 \pm 1.7 ^c
14	81.7 \pm 1.1 ^a	5.8 \pm 0.8 ^c	13.3 \pm 1.1 ^c	30.0 \pm 1.3 ^b	5.8 \pm 1.5 ^c
15	80.8 \pm 0.8 ^a	5.8 \pm 0.8 ^d	16.7 \pm 1.1 ^c	25.8 \pm 0.8 ^b	23.3 \pm 1.1 ^{bc}
MEAN	80.7 \pm 0.7 ^a	4.0 \pm 0.6 ^d	13.9 \pm 0.7 ^c	32.1 \pm 1.3 ^b	22.8 \pm 1.3 ^c

Values with different superscripts within a row are significantly different (P<0.05).

B. Comparison of percentage motility within each treatment group.

Dog	Motility (Mean % \pm SEM)				
	Pre-freeze	10 μ l	50 μ l	100 μ l	200 μ l
1	80.0 \pm 1.8 ^{cdefg}	15.0 \pm 1.8 ^a	24.2 \pm 1.5 ^{ab}	12.5 \pm 1.1 ^g	6.7 \pm 2.1 ^f
2	82.5 \pm 1.1 ^{bcde}	4.2 \pm 1.5 ^b	13.3 \pm 1.7 ^{cdef}	33.3 \pm 1.7 ^{bcd}	10.8 \pm 1.5 ^{ef}
3	78.3 \pm 1.1 ^{defg}	4.2 \pm 1.5 ^b	10.8 \pm 1.5 ^{cdef}	20.0 \pm 1.3 ^{fg}	7.5 \pm 1.7 ^f
4	91.7 \pm 1.1 ^a	2.5 \pm 1.1 ^b	25.0 \pm 1.3 ^a	51.7 \pm 1.7 ^a	23.3 \pm 1.7 ^{cd}
5	87.5 \pm 1.1 ^{abc}	0.8 \pm 0.8 ^b	14.2 \pm 1.5 ^{cde}	35.0 \pm 2.6 ^{bc}	45.8 \pm 1.5 ^a
6	82.5 \pm 1.1 ^{bcde}	0.8 \pm 0.8 ^b	15.8 \pm 1.5 ^{cd}	54.2 \pm 1.5 ^a	34.2 \pm 1.5 ^b
7	88.3 \pm 1.1 ^{ab}	0.0 \pm 0.0 ^b	9.2 \pm 1.5 ^{def}	23.3 \pm 1.1 ^{ef}	27.5 \pm 1.1 ^{bc}
8	73.3 \pm 1.7 ^{fgh}	0.0 \pm 0.0 ^b	6.7 \pm 1.1 ^{ef}	36.7 \pm 2.1 ^{bc}	29.2 \pm 1.5 ^{bc}
9	67.5 \pm 1.1 ^h	0.8 \pm 0.8 ^b	5.8 \pm 0.8 ^f	39.2 \pm 1.5 ^b	24.2 \pm 0.8 ^{cd}
10	82.5 \pm 1.1 ^{bcde}	0.0 \pm 0.0 ^b	10.8 \pm 1.5 ^{cdef}	22.5 \pm 1.1 ^{ef}	19.2 \pm 1.5 ^d
11	75.8 \pm 1.5 ^{efg}	0.8 \pm 0.8 ^b	18.3 \pm 1.1 ^{abc}	19.2 \pm 0.8 ^{fg}	44.2 \pm 1.5 ^a
12	72.5 \pm 1.1 ^{gh}	4.2 \pm 0.8 ^b	9.2 \pm 1.5 ^{def}	29.2 \pm 0.8 ^{cde}	16.2 \pm 1.7 ^{de}
13	85.0 \pm 1.3 ^{abcd}	15.0 \pm 1.3 ^a	15.8 \pm 0.8 ^{cd}	49.2 \pm 1.5 ^a	23.3 \pm 1.7 ^{cd}
14	81.7 \pm 1.1 ^{bcde}	5.8 \pm 0.8 ^b	13.3 \pm 1.1 ^{cdef}	30.0 \pm 1.3 ^{cde}	5.8 \pm 1.5 ^f
15	80.8 \pm 0.8 ^{bcdef}	5.8 \pm 0.8 ^b	16.7 \pm 1.1 ^{bcd}	25.8 \pm 0.8 ^{def}	23.3 \pm 1.1 ^{cd}

Values with different superscripts within a column are significantly different (P<0.05).

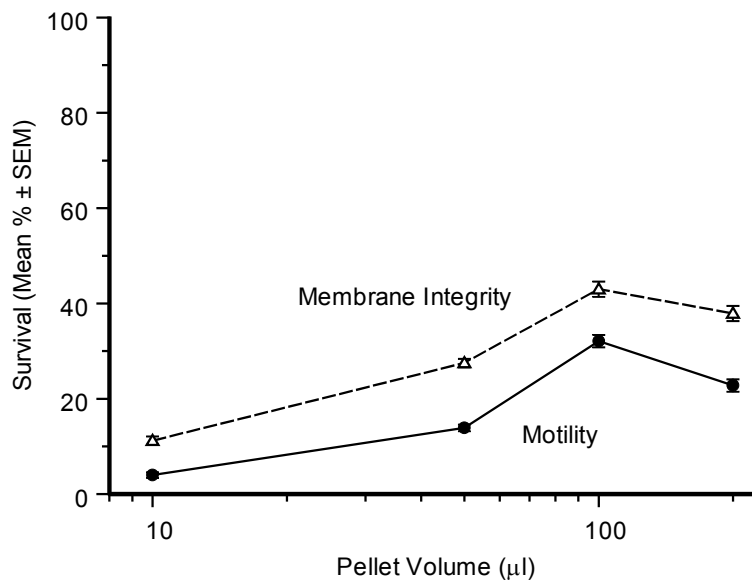


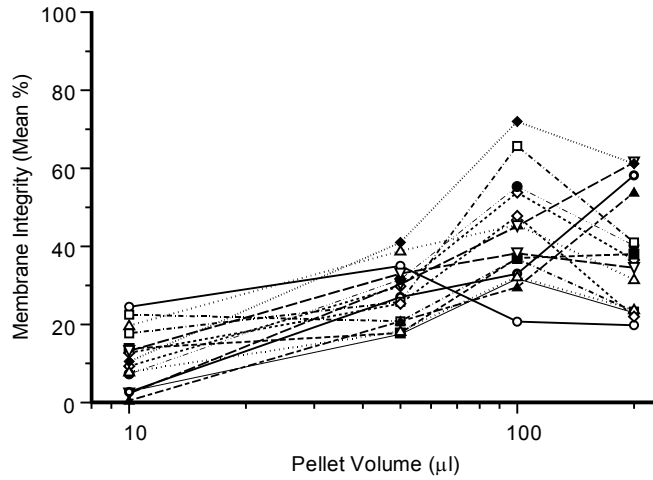
Figure 4.1. Mean percentages of motility and membrane integrity of spermatozoa of 15 dogs plotted as a function of pellet volume. (Note: Pellet volume is plotted on a log scale.)

Because epididymal spermatozoa were used for these experiments, it was only possible to make single observations for each dog with duplicate pellets subjected to each treatment. Each assay of motility or membrane integrity was based on microscopic evaluations of two to six fields of view of each pellet of spermatozoa. Therefore, comparisons of survival of spermatozoa were made for individual dogs treated separately. The mean values of membrane integrity and motility of spermatozoa from each dog for each treatment are shown in Figure 4.2. For spermatozoa cooled as 100 μ l pellets, percentages of membrane integrity varied from ~21% for Dog 1 to ~72% for Dog 9 (Figure 4.3.A), and there were significant differences among the dogs (Table 4.1). Percentages of post-thaw motility of spermatozoa of different dogs varied significantly (Table 4.2), ranging from ~13% for Dog 1 to ~54% for Dog 6 (Figure 4.3.B).

Differences among the 15 dogs in survival of frozen-thawed spermatozoa are also illustrated by the results in Table 4.3. In this table, the pre-freeze values for each of the 15 dogs and the maximum post-thaw values for each dog, as shown in Tables 4.1 and 4.2, are listed. For each dog, the percent survival was calculated by dividing the post-thaw maximum value by the corresponding pre-freeze value. When calculated for motility, survival for the 15 dogs ranged from 25 to 58%. For membrane integrity, survival ranged from 23 to 88%.

The results in Figure 4.4 show the mean values of membrane integrity and motility for each dog. For Dogs 4, 6, and 8, the values for motility and membrane integrity were practically the same. For the other 12 dogs, membrane integrity was greater than motility. This was especially true for spermatozoa from

(A)



(B)

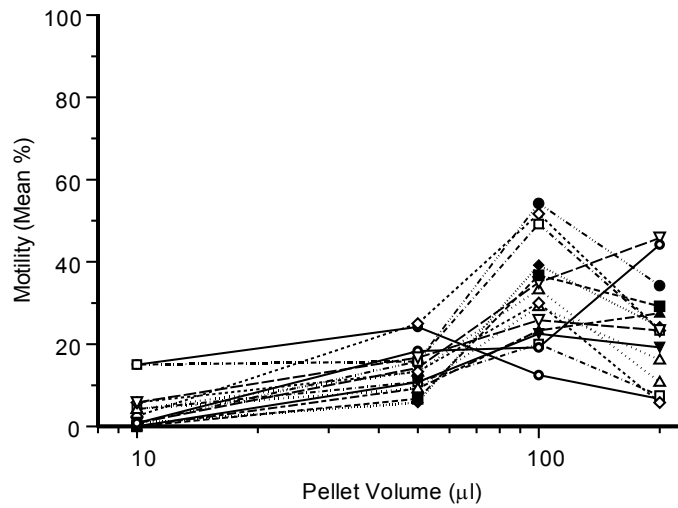
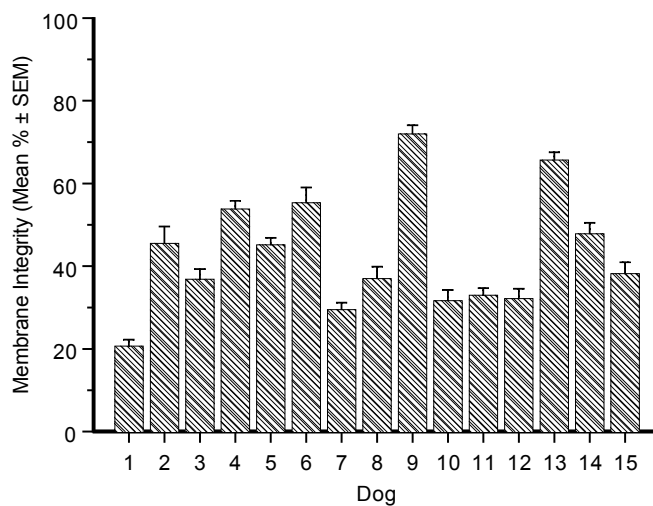


Figure 4.2. Survival of epididymal spermatozoa of individual dogs cooled as pellets of various volumes. (A) Post-thaw membrane integrity. (B) Post-thaw motility. (Note: Pellet volume is plotted on a log scale.)

(A)



(B)

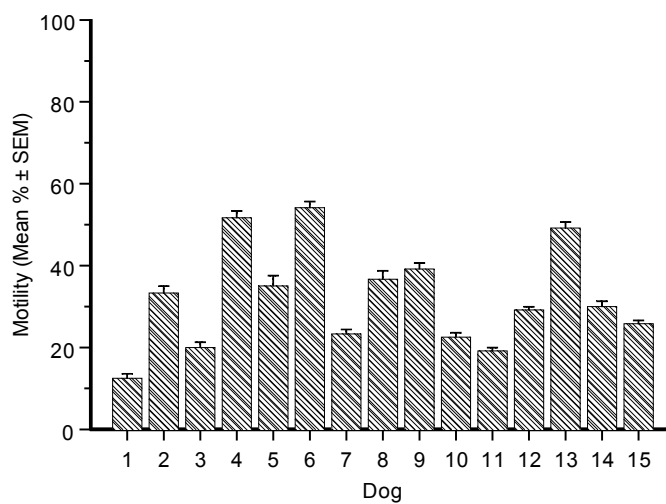


Figure 4.3. Survival of epididymal spermatozoa from individual dogs cooled as pellets of 100 μ l. (A) Membrane Integrity. (B) Motility. The data are the same as those shown in Tables 4.1 and 4.2 for the volume of 100 μ l.

Table 4.3. Maximum post-thaw survival of spermatozoa from individual dogs.

Dog	Motility (Mean % \pm SEM)			Membrane Integrity (Mean % \pm SEM)		
	Pre-freeze control	Post-thaw maximum	% Survival	Pre-freeze control	Post-thaw maximum	% Survival
1	80.0 \pm 1.8	24.2 \pm 1.5	30.3	90.0 \pm 1.4	35.0 \pm 1.9	38.9
2	82.5 \pm 1.1	33.3 \pm 1.7	40.4	90.8 \pm 1.9	33.3 \pm 1.7	36.7
3	78.3 \pm 1.1	20.0 \pm 1.3	25.5	89.0 \pm 2.0	20.0 \pm 1.3	22.5
4	91.7 \pm 1.1	51.7 \pm 1.7	56.4	92.7 \pm 1.2	51.7 \pm 1.7	55.8
5	87.5 \pm 1.1	45.8 \pm 1.5	52.3	92.5 \pm 1.4	61.5 \pm 3.0	66.5
6	82.5 \pm 1.1	54.2 \pm 1.5	65.7	87.2 \pm 1.2	55.3 \pm 3.7	63.4
7	88.3 \pm 1.1	27.5 \pm 1.1	31.1	93.5 \pm 1.9	53.8 \pm 2.7	57.5
8	73.3 \pm 1.7	36.7 \pm 2.1	50.1	78.3 \pm 1.3	38.0 \pm 3.6	48.5
9	67.5 \pm 1.1	39.2 \pm 1.5	58.1	82.2 \pm 2.4	72.0 \pm 2.1	87.6
10	82.5 \pm 1.1	22.5 \pm 1.1	27.3	92.2 \pm 2.4	31.7 \pm 2.6	34.4
11	75.8 \pm 1.5	44.2 \pm 1.5	58.3	84.8 \pm 1.7	58.2 \pm 2.0	68.6
12	72.5 \pm 1.1	29.2 \pm 0.8	40.3	79.3 \pm 2.9	32.2 \pm 2.4	40.6
13	85.0 \pm 1.3	49.2 \pm 1.5	57.9	88.7 \pm 0.9	65.7 \pm 1.9	74.1
14	81.7 \pm 1.1	30.0 \pm 1.3	36.7	86.3 \pm 1.5	47.8 \pm 2.7	55.4
15	80.8 \pm 0.8	25.8 \pm 0.8	31.9	86.2 \pm 1.5	38.2 \pm 2.8	44.3

Pre-freeze control values were mean values for spermatozoa suspended in glycerol held at 4°C throughout the duration of the experimental treatment for each dog. The values of post-thaw motility and membrane integrity are the maxima as a function of pellet volume. Survivals were calculated as percentages of post-thaw values divided by pre-freeze values.

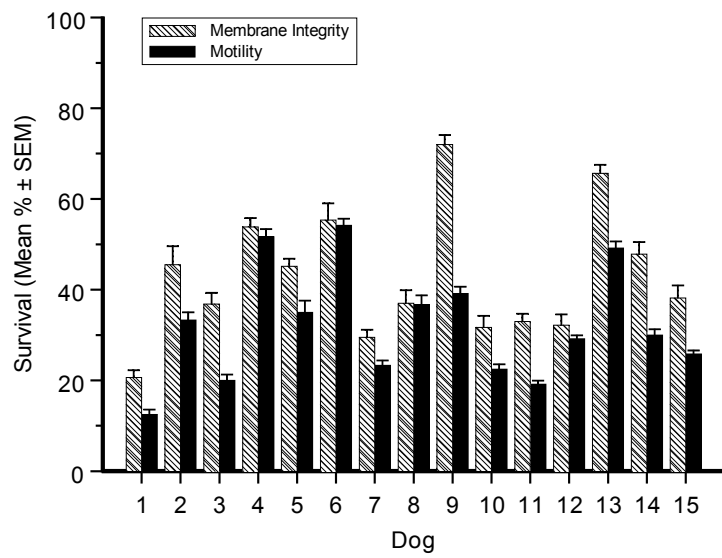


Figure 4.4 Mean values of membrane integrity and motility for spermatozoa of individual dogs cooled as pellets of 100 µl.

dogs 3, 9, 11, 13, 14, and 15. As shown in Figure 4.5, there was a linear correlation between the membrane integrity and motility of all samples of spermatozoa.

According to comparisons by analysis of variance, the age of the dog, the duration of time from surgery to the collection of spermatozoa, and the veterinary clinic from which the testes were obtained had no significant effect on the survival of the spermatozoa ($P>0.05$) (Appendix).

Experiment II

In this experiment, the effect of CPAs and thawing solutions on post-thaw survival of epididymal spermatozoa from individual dogs was determined. Spermatozoa were suspended in 0.43 M glycerol, DMSO, or ethylene glycol, were frozen as 100 μ l pellets, and were thawed in TALP, CCM, or 3% sodium citrate. The membrane integrity and motility of replicate samples of pre-freeze and frozen-thawed spermatozoa are shown in Tables 4.4 and 4.5. Tables 4.4.A and 4.4.B contain the same membrane integrity data, but, in Table 4.4.A, means are compared within each individual dog and, in Table 4.4.B, means are compared within each treatment group. Tables 4.5.A and 4.5.B contain the same motility data; however, in Table 4.5.A, the means are compared within each individual dog and, in Table 4.5.B, means are compared within each treatment group. Fresh samples had significantly higher survival than the cooled and frozen-thawed samples, and the cooled (pre-freeze control) samples had significantly greater survival than the frozen-thawed samples (Tables 4.4 and 4.5). Post-thaw survival was significantly dependent on the CPA, the thawing

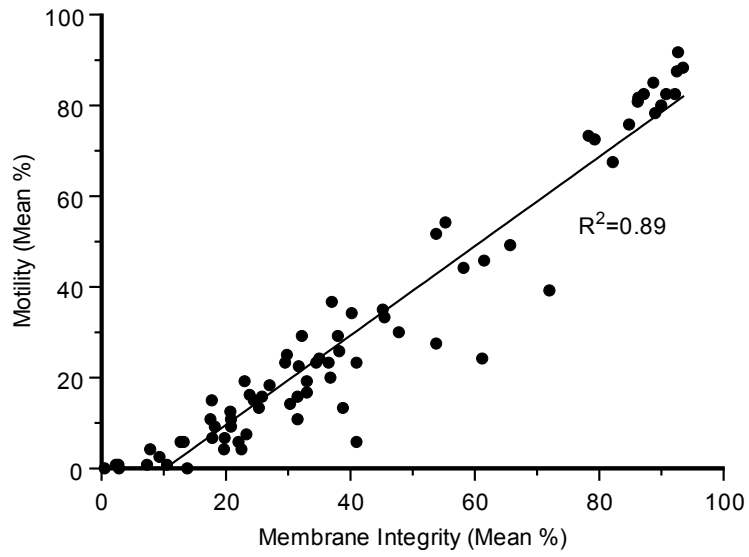


Figure 4.5. Motility of epididymal spermatozoa plotted as a function of membrane integrity of the same epididymal spermatozoa.

Table 4.4 Effect of CPA and thawing solution combinations on membrane integrity of epididymal spermatozoa.
 A. Comparison of percentage membrane integrity within each individual dog.

Dog	Membrane Integrity (Mean % ± SEM)											
	Pre-freeze			CCM			TALP			Na Cit		
	Glycerol	DMSO	EG	Glycerol	DMSO	EG	Glycerol	DMSO	EG	Glycerol	DMSO	EG
1	66.7 ± 2.6 ^a	67.7 ± 2.4 ^a	65.7 ± 4.4 ^a	27.3 ± 2.3 ^b	18.7 ± 3.0 ^b	32.3 ± 3.2 ^b	22.0 ± 2.6 ^b	17.0 ± 2.1 ^b	19.0 ± 4.0 ^b	19.7 ± 2.9 ^b	17.7 ± 3.5 ^b	16.3 ± 2.3 ^b
2	87.3 ± 2.7 ^a	83.7 ± 3.0 ^a	81.0 ± 2.3 ^a	50.3 ± 3.8 ^b	49.0 ± 4.0 ^b	35.3 ± 2.3 ^{bc}	51.0 ± 2.6 ^b	14.0 ± 4.0 ^{cd}	32.3 ± 2.6 ^{bcd}	17.7 ± 2.0 ^{cd}	17.3 ± 1.5 ^{cd}	11.3 ± 1.8 ^d
3	94.7 ± 2.0 ^a	90.0 ± 1.7 ^a	91.3 ± 1.8 ^a	30.7 ± 0.9 ^{bcde}	17.3 ± 2.2 ^{cde}	37.3 ± 2.0 ^{bc}	51.3 ± 2.3 ^b	19.7 ± 1.2 ^{cde}	32.3 ± 2.3 ^{bcd}	16.3 ± 1.8 ^{cde}	13.3 ± 1.9 ^{de}	10.3 ± 2.0 ^e
4	90.0 ± 1.7 ^a	91.0 ± 1.7 ^a	84.3 ± 4.6 ^a	55.3 ± 4.0 ^b	26.0 ± 3.8 ^c	20.7 ± 2.3 ^c	51.0 ± 1.7 ^b	21.3 ± 2.0 ^c	29.0 ± 1.5 ^c	15.7 ± 3.5 ^c	23.0 ± 2.6 ^c	14.3 ± 1.2 ^c
5	93.0 ± 2.6 ^a	81.0 ± 2.5 ^{ab}	91.3 ± 4.2 ^a	64.3 ± 4.1 ^{bc}	28.7 ± 4.5 ^{de}	30.0 ± 1.5 ^{de}	58.0 ± 5.5 ^c	28.0 ± 2.1 ^{de}	25.0 ± 3.8 ^e	47.0 ± 2.1 ^{cd}	21.3 ± 3.3 ^e	28.7 ± 5.0 ^{de}
6	82.3 ± 3.5 ^a	86.0 ± 2.1 ^a	87.0 ± 4.4 ^a	39.7 ± 2.3 ^{bcd}	17.7 ± 2.7 ^e	45.3 ± 4.3 ^b	40.3 ± 1.8 ^{bc}	16.7 ± 2.0 ^e	23.7 ± 4.3 ^{cde}	18.7 ± 2.3 ^{de}	18.3 ± 4.5 ^{de}	34.0 ± 4.0 ^{bcd}
7	92.3 ± 1.8 ^a	85.0 ± 4.2 ^a	84.7 ± 3.2 ^a	25.3 ± 3.8 ^b	16.0 ± 3.2 ^b	17.3 ± 3.0 ^b	25.0 ± 3.5 ^b	24.3 ± 2.4 ^b	27.7 ± 4.4 ^b	26.7 ± 4.6 ^b	12.3 ± 2.0 ^b	11.0 ± 2.3 ^b
8	90.7 ± 1.5 ^a	92.3 ± 2.0 ^a	92.0 ± 2.5 ^a	27.7 ± 2.3 ^{bcd}	31.0 ± 6.1 ^{bcd}	38.7 ± 1.2 ^{bc}	41.3 ± 4.6 ^b	24.3 ± 3.5 ^{bcd}	26.7 ± 2.6 ^{bcd}	17.7 ± 2.0 ^{cd}	14.3 ± 1.8 ^d	15.3 ± 3.0 ^d
9	80.7 ± 2.2 ^a	81.3 ± 2.7 ^a	85.7 ± 3.8 ^a	50.7 ± 3.2 ^b	27.3 ± 5.0 ^c	33.3 ± 2.6 ^{bc}	34.3 ± 3.5 ^{bc}	31.0 ± 2.0 ^{bc}	27.3 ± 2.4 ^c	24.3 ± 3.8 ^c	19.7 ± 2.4 ^c	16.0 ± 2.9 ^c
10	86.0 ± 2.1 ^a	88.0 ± 2.5 ^a	91.7 ± 2.0 ^a	31.3 ± 2.4 ^b	32.3 ± 2.7 ^b	33.3 ± 4.6 ^b	24.7 ± 3.8 ^b	22.3 ± 2.3 ^b	24.0 ± 5.5 ^b	20.0 ± 1.5 ^b	18.0 ± 2.3 ^b	21.3 ± 1.5 ^b
11	91.3 ± 1.5 ^a	94.7 ± 1.5 ^a	93.0 ± 3.2 ^a	43.7 ± 3.2 ^b	33.0 ± 3.2 ^b	39.3 ± 4.3 ^b	35.7 ± 4.1 ^b	29.3 ± 2.2 ^b	25.0 ± 4.6 ^b	27.7 ± 4.3 ^b	23.7 ± 1.2 ^b	24.7 ± 5.6 ^b
12	87.3 ± 1.9 ^a	89.7 ± 4.1 ^a	82.7 ± 2.0 ^a	37.0 ± 2.3 ^b	23.3 ± 5.0 ^b	36.0 ± 3.8 ^b	28.3 ± 3.8 ^b	20.7 ± 2.2 ^b	30.0 ± 2.1 ^b	23.3 ± 3.5 ^b	21.7 ± 3.2 ^b	18.0 ± 3.2 ^b
13	83.7 ± 3.8 ^a	80.7 ± 3.4 ^a	83.7 ± 5.5 ^a	40.7 ± 2.6 ^b	15.7 ± 4.8 ^c	19.3 ± 3.8 ^{bc}	19.3 ± 2.0 ^{bc}	19.7 ± 2.3 ^{bc}	19.0 ± 4.6 ^c	19.3 ± 1.2 ^{bc}	17.7 ± 2.3 ^c	12.3 ± 2.3 ^c
14	85.3 ± 3.3 ^a	76.3 ± 3.8 ^{ab}	86.7 ± 3.5 ^a	60.7 ± 5.5 ^{bc}	34.7 ± 4.1 ^{de}	45.0 ± 5.1 ^{cd}	54.0 ± 7.0 ^{cd}	20.7 ± 1.5 ^e	45.7 ± 3.8 ^{cd}	45.3 ± 4.1 ^{cd}	17.0 ± 2.6 ^e	44.0 ± 5.1 ^{cd}
15	94.7 ± 1.5 ^a	83.3 ± 2.3 ^a	84.3 ± 3.5 ^a	42.0 ± 3.5 ^{bc}	41.3 ± 3.5 ^{bc}	25.3 ± 4.3 ^{cd}	33.7 ± 3.5 ^{bcd}	27.0 ± 0.6 ^{cd}	51.3 ± 5.2 ^b	22.3 ± 2.0 ^{cd}	18.7 ± 3.5 ^d	13.3 ± 4.5 ^d
16	90.7 ± 2.4 ^a	88.7 ± 4.9 ^a	92.0 ± 1.5 ^a	42.0 ± 2.6 ^b	35.3 ± 4.8 ^{bc}	44.7 ± 4.1 ^b	28.7 ± 1.8 ^{bc}	28.7 ± 3.8 ^{bc}	40.7 ± 5.5 ^b	16.0 ± 2.9 ^c	35.0 ± 2.6 ^{bc}	14.7 ± 3.5 ^c
MEAN	87.3 ± 1.1 ^a	85.0 ± 1 ^a	86.1 ± 2.0 ^a	41.8 ± 1.8 ^b	28.0 ± 1.6 ^{cde}	33.3 ± 1.4 ^{bc}	37.4 ± 2 ^{bc}	22.8 ± 1 ^{def}	30.0 ± 2 ^{cd}	23.6 ± 2 ^{def}	19.3 ± 1 ^{ef}	19.1 ± 2 ^f

Values with different superscripts within a row are significantly different (P<0.05).

Table 4.4 (continued)

B. Comparison of percentage membrane integrity within each treatment group.

Dog	Membrane Integrity (Mean % ± SEM)											
	Pre-freeze			CCM			TALP			Na Cit		
	Glycerol	DMSO	EG	Glycerol	DMSO	EG	Glycerol	DMSO	EG	Glycerol	DMSO	EG
1	66.7 ± 2.6 ^b	67.7 ± 2.4 ^b	65.7 ± 4.4 ^b	27.3 ± 2.3 ^e	18.7 ± 3.0 ^c	32.3 ± 3.2 ^{abc}	22.0 ± 2.6 ^{cd}	17.0 ± 2.1 ^a	19.0 ± 4.0 ^d	19.7 ± 2.9 ^c	17.7 ± 3.5 ^{ab}	16.3 ± 2.3 ^{bc}
2	87.3 ± 2.7 ^{ab}	83.7 ± 3.0 ^{ab}	81.0 ± 2.3 ^{ab}	50.3 ± 3.8 ^{abcd}	49.0 ± 4.0 ^a	35.3 ± 2.3 ^{abc}	51.0 ± 2.6 ^{ab}	14.0 ± 4.0 ^a	32.3 ± 2.6 ^{abcd}	17.7 ± 2.0 ^c	17.3 ± 1.5 ^{ab}	11.3 ± 1.8 ^c
3	94.7 ± 2.0 ^a	90.0 ± 1.7 ^a	91.3 ± 1.8 ^a	30.7 ± 0.9 ^{de}	17.3 ± 2.2 ^c	37.3 ± 2.0 ^{abc}	51.3 ± 2.3 ^{ab}	19.7 ± 1.2 ^a	32.3 ± 2.3 ^{abcd}	16.3 ± 1.8 ^c	13.3 ± 1.9 ^b	10.3 ± 2.0 ^c
4	90.0 ± 1.7 ^a	91.0 ± 1.7 ^a	84.3 ± 4.6 ^{ab}	55.3 ± 4.0 ^{abc}	26.0 ± 3.8 ^{bc}	20.7 ± 2.3 ^{bc}	51.0 ± 1.7 ^{ab}	21.3 ± 2.0 ^a	29.0 ± 1.5 ^{bcd}	15.7 ± 3.5 ^c	23.0 ± 2.6 ^{ab}	14.3 ± 1.2 ^{bc}
5	93.0 ± 2.6 ^a	81.0 ± 2.5 ^{ab}	91.3 ± 4.2 ^a	64.3 ± 4.1 ^a	28.7 ± 4.5 ^{abc}	30.0 ± 1.5 ^{abc}	58.0 ± 5.5 ^a	28.0 ± 2.1 ^a	25.0 ± 3.8 ^{bcd}	47.0 ± 2.1 ^a	21.3 ± 3.3 ^{ab}	28.7 ± 5.0 ^{abc}
6	82.3 ± 3.5 ^{ab}	86.0 ± 2.1 ^{ab}	87.0 ± 4.4 ^{ab}	39.7 ± 2.3 ^{bcde}	17.7 ± 2.7 ^c	45.3 ± 4.3 ^a	40.3 ± 1.8 ^{abcd}	16.7 ± 2.0 ^a	23.7 ± 4.3 ^{cd}	18.7 ± 2.3 ^c	18.3 ± 4.5 ^{ab}	34.0 ± 4.0 ^{ab}
7	92.3 ± 1.8 ^a	85.0 ± 4.2 ^{ab}	84.7 ± 3.2 ^{ab}	25.3 ± 3.8 ^e	16.0 ± 3.2 ^c	17.3 ± 3.0 ^c	25.0 ± 3.5 ^{cd}	24.3 ± 2.4 ^a	27.7 ± 4.4 ^{bcd}	26.7 ± 4.6 ^{abc}	12.3 ± 2.0 ^b	11.0 ± 2.3 ^c
8	90.7 ± 1.5 ^a	92.3 ± 2.0 ^a	92.0 ± 2.5 ^a	27.7 ± 2.3 ^e	31.0 ± 6.1 ^{abc}	38.7 ± 1.2 ^{abc}	41.3 ± 4.6 ^{abc}	24.3 ± 3.5 ^a	26.7 ± 2.6 ^{bcd}	17.7 ± 2.0 ^c	14.3 ± 1.8 ^{ab}	15.3 ± 3.0 ^{bc}
9	80.7 ± 2.2 ^{ab}	81.3 ± 2.7 ^{ab}	85.7 ± 3.8 ^{ab}	50.7 ± 3.2 ^{abcd}	27.3 ± 5.0 ^{bc}	33.3 ± 2.6 ^{abc}	34.3 ± 3.5 ^{bcd}	31.0 ± 2.0 ^a	27.3 ± 2.4 ^{bcd}	24.3 ± 3.8 ^{bc}	19.7 ± 2.4 ^{ab}	16.0 ± 2.9 ^{bc}
10	86.0 ± 2.1 ^{ab}	88.0 ± 2.5 ^{ab}	91.7 ± 2.0 ^a	31.3 ± 2.4 ^{de}	32.3 ± 2.7 ^{abc}	33.3 ± 4.6 ^{abc}	24.7 ± 3.8 ^{cd}	22.3 ± 2.3 ^a	24.0 ± 5.5 ^{cd}	20.0 ± 1.5 ^c	18.0 ± 2.3 ^{ab}	21.3 ± 1.5 ^{bc}
11	91.3 ± 1.5 ^a	94.7 ± 1.5 ^a	93.0 ± 3.2 ^a	43.7 ± 3.2 ^{abcde}	33.0 ± 3.2 ^{abc}	39.3 ± 4.3 ^{ab}	35.7 ± 4.1 ^{bcd}	29.3 ± 2.2 ^a	25.0 ± 4.6 ^{bcd}	27.7 ± 4.3 ^{abc}	23.7 ± 1.2 ^{ab}	24.7 ± 5.6 ^{abc}
12	87.3 ± 1.9 ^{ab}	89.7 ± 4.1 ^a	82.7 ± 2.0 ^{ab}	37.0 ± 2.3 ^{ode}	23.3 ± 5.0 ^{bc}	36.0 ± 3.8 ^{abc}	28.3 ± 3.8 ^{cd}	20.7 ± 2.2 ^a	30.0 ± 2.1 ^{abcd}	23.3 ± 3.5 ^c	21.7 ± 3.2 ^{ab}	18.0 ± 3.2 ^{bc}
13	83.7 ± 3.8 ^{ab}	80.7 ± 3.4 ^{ab}	83.7 ± 5.5 ^{ab}	40.7 ± 2.6 ^{bcde}	15.7 ± 4.8 ^c	19.3 ± 3.8 ^{bc}	19.3 ± 2.0 ^d	19.7 ± 2.3 ^a	19.0 ± 4.6 ^d	19.3 ± 1.2 ^c	17.7 ± 2.3 ^{ab}	12.3 ± 2.3 ^c
14	85.3 ± 3.3 ^{ab}	76.3 ± 3.8 ^{ab}	86.7 ± 3.5 ^{ab}	60.7 ± 5.5 ^{ab}	34.7 ± 4.1 ^{abc}	45.0 ± 5.1 ^a	54.0 ± 7.0 ^{ab}	20.7 ± 1.5 ^a	45.7 ± 3.8 ^{ab}	45.3 ± 4.1 ^{ab}	17.0 ± 2.6 ^{ab}	44.0 ± 5.1 ^a
15	94.7 ± 1.5 ^a	83.3 ± 2.3 ^{ab}	84.3 ± 3.5 ^{ab}	42.0 ± 3.5 ^{bcde}	41.3 ± 3.5 ^{ab}	25.3 ± 4.3 ^{abc}	33.7 ± 3.5 ^{bcd}	27.0 ± 0.6 ^a	51.3 ± 5.2 ^a	22.3 ± 2.0 ^c	18.7 ± 3.5 ^{ab}	13.3 ± 4.5 ^{bc}
16	90.7 ± 2.4 ^a	88.7 ± 4.9 ^{ab}	92.0 ± 1.5 ^a	42.0 ± 2.6 ^{bcde}	35.3 ± 4.8 ^{abc}	44.7 ± 4.1 ^a	28.7 ± 1.8 ^{cd}	28.7 ± 3.8 ^a	40.7 ± 5.5 ^{abc}	16.0 ± 2.9 ^c	35.0 ± 2.6 ^a	14.7 ± 3.5 ^{bc}

Values with different superscripts within a column are significantly different (P<0.05).

Table 4.5. Effect of CPA and thawing solution combinations on motility of epididymal spermatozoa.
A. Comparison of percentage motility within each individual dog.

Dog	Motility (Mean % \pm SEM)											
	Pre-freeze			CCM			TALP			Na Cit		
	Glycerol	DMSO	EG	Glycerol	DMSO	EG	Glycerol	DMSO	EG	Glycerol	DMSO	EG
1	51.7 \pm 1.7 ^a	53.3 \pm 1.7 ^a	50.0 \pm 2.9 ^a	13.3 \pm 1.7 ^b	3.3 \pm 1.7 ^c	16.7 \pm 1.7 ^b	8.3 \pm 1.7 ^{bc}	1.7 \pm 1.7 ^c	3.3 \pm 1.7 ^c	0.0 \pm 0.0 ^c	0.0 \pm 0.0 ^c	0.0 \pm 0.0 ^c
2	71.7 \pm 1.7 ^a	68.3 \pm 1.7 ^a	70.0 \pm 2.9 ^a	33.3 \pm 1.7 ^b	35.0 \pm 0.0 ^b	21.7 \pm 1.7 ^{cd}	28.3 \pm 1.7 ^{bc}	1.7 \pm 1.7 ^e	16.7 \pm 1.7 ^d	1.7 \pm 1.7 ^e	3.3 \pm 1.7 ^e	0.0 \pm 0.0 ^e
3	83.3 \pm 1.7 ^a	80.0 \pm 0.0 ^a	81.7 \pm 1.7 ^a	11.7 \pm 1.7 ^{ode}	8.3 \pm 1.7 ^{def}	18.3 \pm 1.7 ^{bc}	21.7 \pm 1.7 ^b	6.7 \pm 1.7 ^{def}	13.3 \pm 1.7 ^{bcd}	1.7 \pm 1.7 ^f	3.3 \pm 1.7 ^{ef}	0.0 \pm 0.0 ^f
4	83.3 \pm 1.7 ^a	88.3 \pm 1.7 ^a	81.7 \pm 1.7 ^a	40.0 \pm 2.9 ^b	13.3 \pm 1.7 ^{cd}	8.3 \pm 1.7 ^{ode}	36.7 \pm 1.7 ^b	6.7 \pm 1.7 ^{de}	16.7 \pm 1.7 ^c	1.7 \pm 1.7 ^e	8.3 \pm 1.7 ^{cde}	5.0 \pm 0.0 ^{de}
5	85.0 \pm 0.0 ^a	78.3 \pm 1.7 ^a	78.3 \pm 1.7 ^a	48.3 \pm 1.7 ^b	10.0 \pm 0.0 ^{ef}	16.7 \pm 1.7 ^{def}	36.7 \pm 1.7 ^c	11.7 \pm 1.7 ^{def}	16.7 \pm 1.7 ^{def}	20.0 \pm 0.0 ^d	8.3 \pm 1.7 ^f	18.3 \pm 1.7 ^{de}
6	76.7 \pm 1.7 ^a	71.7 \pm 1.7 ^a	75.0 \pm 0.0 ^a	23.3 \pm 1.7 ^b	8.3 \pm 1.7 ^{cd}	26.7 \pm 1.7 ^b	20.0 \pm 0.0 ^b	3.3 \pm 1.7 ^{cd}	8.3 \pm 1.7 ^{cd}	0.0 \pm 0.0 ^d	0.0 \pm 0.0 ^d	10.0 \pm 0.0 ^c
7	83.3 \pm 1.7 ^a	66.7 \pm 1.7 ^b	68.3 \pm 1.7 ^b	10.0 \pm 0.0 ^{cd}	6.7 \pm 1.7 ^{cd}	8.3 \pm 1.7 ^{cd}	11.7 \pm 1.7 ^{cd}	10.0 \pm 0.0 ^{cd}	13.3 \pm 1.7 ^c	8.3 \pm 1.7 ^{cd}	5.0 \pm 0.0 ^{cd}	3.3 \pm 1.7 ^d
8	73.3 \pm 1.7 ^a	81.7 \pm 1.7 ^a	81.7 \pm 1.7 ^a	15.0 \pm 0.0 ^{bcd}	13.3 \pm 1.7 ^{bcde}	18.3 \pm 1.7 ^{bc}	21.7 \pm 1.7 ^b	8.3 \pm 1.7 ^{defg}	11.7 \pm 1.7 ^{cdef}	3.3 \pm 1.7 ^{fg}	0.0 \pm 0.0 ^g	5.0 \pm 0.0 ^{efg}
9	63.3 \pm 1.7 ^a	61.7 \pm 1.7 ^a	65.0 \pm 0.0 ^a	26.7 \pm 1.7 ^b	10.0 \pm 0.0 ^{cde}	11.7 \pm 1.7 ^{cd}	16.7 \pm 1.7 ^c	11.7 \pm 1.7 ^{cd}	8.3 \pm 1.7 ^{cde}	6.7 \pm 1.7 ^{de}	3.3 \pm 1.7 ^{de}	1.7 \pm 1.7 ^e
10	76.7 \pm 1.7 ^a	70.0 \pm 0.0 ^a	78.3 \pm 1.7 ^a	13.3 \pm 1.7 ^{bc}	8.3 \pm 1.7 ^{bcde}	15.0 \pm 0.0 ^b	10.0 \pm 0.0 ^{bcd}	5.0 \pm 0.0 ^{cde}	8.3 \pm 1.7 ^{bcde}	1.7 \pm 1.7 ^{de}	0.0 \pm 0.0 ^e	5.0 \pm 0.0 ^{cde}
11	85.0 \pm 0.0 ^a	80.0 \pm 0.0 ^a	83.3 \pm 1.7 ^a	23.3 \pm 1.7 ^b	18.3 \pm 1.7 ^{bc}	11.7 \pm 1.7 ^{cd}	18.3 \pm 1.7 ^{bc}	11.7 \pm 1.7 ^{cd}	8.3 \pm 1.7 ^d	8.3 \pm 1.7 ^d	6.7 \pm 1.7 ^d	6.7 \pm 1.7 ^d
12	76.7 \pm 1.7 ^a	78.3 \pm 1.7 ^a	80.0 \pm 2.9 ^a	8.3 \pm 1.7 ^{bc}	0.0 \pm 0.0 ^c	10.0 \pm 2.9 ^b	6.7 \pm 1.7 ^{bc}	0.0 \pm 0.0 ^c	8.3 \pm 1.7 ^{bc}	1.7 \pm 1.7 ^{bc}	0.0 \pm 0.0 ^c	0.0 \pm 0.0 ^c
13	66.7 \pm 1.7 ^a	66.7 \pm 1.7 ^a	68.3 \pm 1.7 ^a	15.0 \pm 0.0 ^b	0.0 \pm 0.0 ^c	3.3 \pm 1.7 ^c	6.7 \pm 1.7 ^{bc}	1.7 \pm 1.7 ^c	1.7 \pm 1.7 ^c	3.3 \pm 1.7 ^c	0.0 \pm 0.0 ^c	0.0 \pm 0.0 ^c
14	71.7 \pm 1.7 ^a	66.7 \pm 1.7 ^a	71.7 \pm 1.7 ^a	31.7 \pm 1.7 ^b	13.3 \pm 1.7 ^{ef}	20.0 \pm 0.0 ^{cde}	28.3 \pm 1.7 ^{bc}	5.0 \pm 0.0 ^{fg}	23.3 \pm 1.7 ^{bcd}	18.3 \pm 1.7 ^{de}	3.3 \pm 1.7 ^g	18.3 \pm 1.7 ^{de}
15	81.7 \pm 1.7 ^a	68.3 \pm 1.7 ^b	66.7 \pm 1.7 ^b	25.0 \pm 0.0 ^c	13.3 \pm 1.7 ^d	11.7 \pm 1.7 ^d	13.3 \pm 1.7 ^d	10.0 \pm 2.9 ^{de}	23.3 \pm 1.7 ^c	6.7 \pm 1.7 ^{def}	1.7 \pm 1.7 ^{ef}	0.0 \pm 0.0 ^f
16	83.3 \pm 1.7 ^a	81.7 \pm 1.7 ^{ab}	73.3 \pm 1.7 ^b	18.3 \pm 1.7 ^c	11.7 \pm 1.7 ^{cd}	11.7 \pm 1.7 ^{cd}	10.0 \pm 0.0 ^{cde}	8.3 \pm 1.7 ^{de}	11.7 \pm 1.7 ^{cd}	1.7 \pm 1.7 ^e	10.0 \pm 0.0 ^{cde}	5.0 \pm 0.0 ^{de}
MEAN	75.8 \pm 1.4 ^a	72.6 \pm 1.3 ^a	73.3 \pm 1.3 ^a	22.3 \pm 1.7 ^b	10.8 \pm 1.2 ^{cde}	14.4 \pm 0.9 ^{cd}	18.4 \pm 1 ^{bc}	6.5 \pm 1 ^{ef}	12.1 \pm 1 ^{de}	5.3 \pm 1 ^{ef}	3.3 \pm 1 ^f	4.9 \pm 1 ^{ef}

Values with different superscripts within a row are significantly different (P<0.05).

Table 4.5 (continued)

B. Comparison of percentage motility within each treatment group.

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Dog	Motility (Mean % ± SEM)											
	Pre-freeze			CCM			TALP			Na Cit		
	Glycerol	DMSO	EG	Glycerol	DMSO	EG	Glycerol	DMSO	EG	Glycerol	DMSO	EG
1	51.7 ± 1.7 ^f	53.3 ± 1.7 ^g	50.0 ± 2.9 ^g	13.3 ± 1.7 ^{gh}	3.3 ± 1.7 ^{de}	16.7 ± 1.7 ^{bode}	8.3 ± 1.7 ^{fg}	1.7 ± 1.7 ^{bc}	3.3 ± 1.7 ^{cd}	0.0 ± 0.0 ^b	0.0 ± 0.0 ^b	0.0 ± 0.0 ^c
2	71.7 ± 1.7 ^{cde}	68.3 ± 1.7 ^{ef}	70.0 ± 2.9 ^{def}	33.3 ± 1.7 ^{bc}	35.0 ± 0.0 ^a	21.7 ± 1.7 ^{ab}	28.3 ± 1.7 ^{ab}	1.7 ± 1.7 ^{bc}	16.7 ± 1.7 ^{ab}	1.7 ± 1.7 ^b	3.3 ± 1.7 ^{ab}	0.0 ± 0.0 ^c
3	83.3 ± 1.7 ^a	80.0 ± 0.0 ^{abc}	81.7 ± 1.7 ^{ab}	11.7 ± 1.7 ^{gh}	8.3 ± 1.7 ^{cde}	18.3 ± 1.7 ^{abcd}	21.7 ± 1.7 ^{bc}	6.7 ± 1.7 ^{abc}	13.3 ± 1.7 ^b	1.7 ± 1.7 ^b	3.3 ± 1.7 ^{ab}	0.0 ± 0.0 ^c
4	83.3 ± 1.7 ^a	88.3 ± 1.7 ^a	81.7 ± 1.7 ^{ab}	40.0 ± 2.9 ^{ab}	13.3 ± 1.7 ^{bc}	8.3 ± 1.7 ^{ef}	36.7 ± 1.7 ^a	6.7 ± 1.7 ^{abc}	16.7 ± 1.7 ^{ab}	1.7 ± 1.7 ^b	8.3 ± 1.7 ^{ab}	5.0 ± 0.0 ^{bc}
5	85.0 ± 0.0 ^a	78.3 ± 1.7 ^{bcd}	78.3 ± 1.7 ^{abcd}	48.3 ± 1.7 ^a	10.0 ± 0.0 ^{bcd}	16.7 ± 1.7 ^{bode}	36.7 ± 1.7 ^a	11.7 ± 1.7 ^a	16.7 ± 1.7 ^{ab}	20.0 ± 0.0 ^a	8.3 ± 1.7 ^{ab}	18.3 ± 1.7 ^a
6	76.7 ± 1.7 ^{abc}	71.7 ± 1.7 ^{cde}	75.0 ± 0.0 ^{abcde}	23.3 ± 1.7 ^{def}	8.3 ± 1.7 ^{cde}	26.7 ± 1.7 ^a	20.0 ± 0.0 ^{bcd}	3.3 ± 1.7 ^{abc}	8.3 ± 1.7 ^{bcd}	0.0 ± 0.0 ^b	0.0 ± 0.0 ^b	10.0 ± 0.0 ^{ab}
7	83.3 ± 1.7 ^a	66.7 ± 1.7 ^{ef}	68.3 ± 1.7 ^{ef}	10.0 ± 0.0 ^{gh}	6.7 ± 1.7 ^{cde}	8.3 ± 1.7 ^{ef}	11.7 ± 1.7 ^{defg}	10.0 ± 0.0 ^{ab}	13.3 ± 1.7 ^b	8.3 ± 1.7 ^b	5.0 ± 0.0 ^{ab}	3.3 ± 1.7 ^{bc}
8	73.3 ± 1.7 ^{bcd}	81.7 ± 1.7 ^{ab}	81.7 ± 1.7 ^{ab}	15.0 ± 0.0 ^{fgh}	13.3 ± 1.7 ^{bc}	18.3 ± 1.7 ^{abcd}	21.7 ± 1.7 ^{bc}	8.3 ± 1.7 ^{abc}	11.7 ± 1.7 ^{bc}	3.3 ± 1.7 ^b	0.0 ± 0.0 ^b	5.0 ± 0.0 ^{bc}
9	63.3 ± 1.7 ^e	61.7 ± 1.7 ^{fg}	65.0 ± 0.0 ^f	26.7 ± 1.7 ^{cde}	10.0 ± 0.0 ^{bcd}	11.7 ± 1.7 ^{cdef}	16.7 ± 1.7 ^{cdef}	11.7 ± 1.7 ^a	8.3 ± 1.7 ^{bcd}	6.7 ± 1.7 ^b	3.3 ± 1.7 ^{ab}	1.7 ± 1.7 ^{bc}
10	76.7 ± 1.7 ^{abc}	70.0 ± 0.0 ^{def}	78.3 ± 1.7 ^{abcd}	13.3 ± 1.7 ^{gh}	8.3 ± 1.7 ^{cde}	15.0 ± 0.0 ^{bode}	10.0 ± 0.0 ^{efg}	5.0 ± 0.0 ^{abc}	8.3 ± 1.7 ^{bcd}	1.7 ± 1.7 ^b	0.0 ± 0.0 ^b	5.0 ± 0.0 ^{bc}
11	85.0 ± 0.0 ^a	80.0 ± 0.0 ^{abc}	83.3 ± 1.7 ^a	23.3 ± 1.7 ^{def}	18.3 ± 1.7 ^b	11.7 ± 1.7 ^{cdef}	18.3 ± 1.7 ^{cde}	11.7 ± 1.7 ^a	8.3 ± 1.7 ^{bcd}	8.3 ± 1.7 ^b	6.7 ± 1.7 ^{ab}	6.7 ± 1.7 ^{bc}
12	76.7 ± 1.7 ^{abc}	78.3 ± 1.7 ^{bcd}	80.0 ± 2.9 ^{abc}	8.3 ± 1.7 ^h	0.0 ± 0.0 ^e	10.0 ± 2.9 ^{def}	6.7 ± 1.7 ^g	0.0 ± 0.0 ^c	8.3 ± 1.7 ^{bcd}	1.7 ± 1.7 ^b	0.0 ± 0.0 ^b	0.0 ± 0.0 ^c
13	66.7 ± 1.7 ^{de}	66.7 ± 1.7 ^{ef}	68.3 ± 1.7 ^{ef}	15.0 ± 0.0 ^{fgh}	0.0 ± 0.0 ^e	3.3 ± 1.7 ^f	6.7 ± 1.7 ^g	1.7 ± 1.7 ^{bc}	1.7 ± 1.7 ^d	3.3 ± 1.7 ^b	0.0 ± 0.0 ^b	0.0 ± 0.0 ^c
14	71.7 ± 1.7 ^{cde}	66.7 ± 1.7 ^{ef}	71.7 ± 1.7 ^{cdef}	31.7 ± 1.7 ^{bcd}	13.3 ± 1.7 ^{bc}	20.0 ± 0.0 ^{abc}	28.3 ± 1.7 ^{ab}	5.0 ± 0.0 ^{abc}	23.3 ± 1.7 ^a	18.3 ± 1.7 ^a	3.3 ± 1.7 ^{ab}	18.3 ± 1.7 ^a
15	81.7 ± 1.7 ^{ab}	68.3 ± 1.7 ^{ef}	66.7 ± 1.7 ^{ef}	25.0 ± 0.0 ^{cde}	13.3 ± 1.7 ^{bc}	11.7 ± 1.7 ^{cdef}	13.3 ± 1.7 ^{cdefg}	10.0 ± 2.9 ^{ab}	23.3 ± 1.7 ^a	6.7 ± 1.7 ^b	1.7 ± 1.7 ^{ab}	0.0 ± 0.0 ^c
16	83.3 ± 1.7 ^a	81.7 ± 1.7 ^{ab}	73.3 ± 1.7 ^{bcd}	18.3 ± 1.7 ^{efg}	11.7 ± 1.7 ^{bcd}	11.7 ± 1.7 ^{cdef}	10.0 ± 0.0 ^{efg}	8.3 ± 1.7 ^{abc}	11.7 ± 1.7 ^{bc}	1.7 ± 1.7 ^b	10.0 ± 0.0 ^a	5.0 ± 0.0 ^{bc}

Values with different superscripts within a column are significantly different (P<0.05).

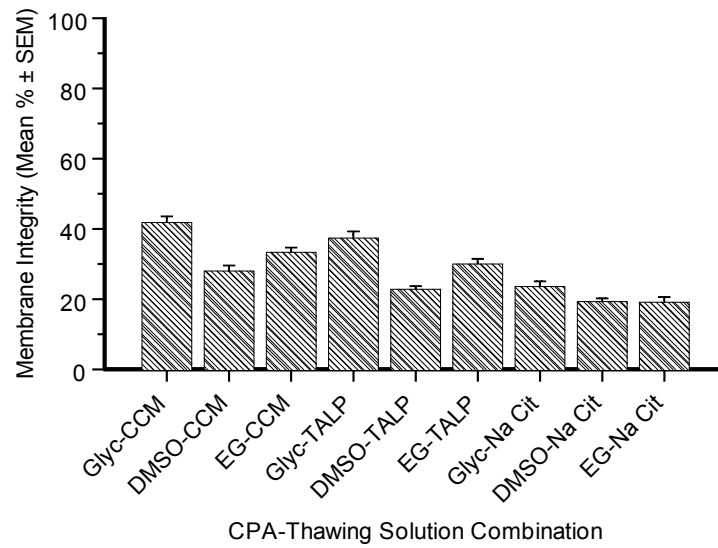
solution, and the interaction between CPA and thawing solution. The combinations glycerol-CCM, glycerol-TALP, and EG-CCM exhibited the highest mean values of membrane integrity of 42%, 37%, and 33%, respectively ($P<0.05$) (Figure 4.6.A). The CPA-thawing solution combinations that exhibited the highest values of motility were glycerol-CCM and glycerol-TALP, with values of 22% and 18%, respectively ($P<0.05$) (Figure 4.6.B).

It was only possible to make single observations for each dog with a single pellet subjected to each combination of treatments. Each assay of motility or membrane integrity was based on microscopic observations of two to six fields of view of each pellet of spermatozoa. Comparisons of survival of spermatozoa were made from individual dogs treated separately. The post-thaw survival of epididymal spermatozoa treated with the CPA glycerol and thawed in CCM from each dog is shown in Figure 4.7. For spermatozoa treated with the CPA glycerol and thawed in CCM, "survival" assayed by membrane integrity varied from 25% for Dog 7 to 64% for Dog 5 (Figure 4.7.A), and there were significant differences among individual dogs (Table 4.4). Motility varied significantly among individual dogs (Table 4.5), from 8% for Dog 12 to 48% for Dog 5 (Figure 4.7.B).

The results in Figure 4.8 show the mean values of membrane integrity and motility of spermatozoa treated with the CPA glycerol and thawed in CCM for each dog. For all dogs, membrane integrity was greater than motility.

As shown in Figure 4.9, there was a linear correlation between the membrane integrity and motility of all samples of spermatozoa.

(A)



(B)

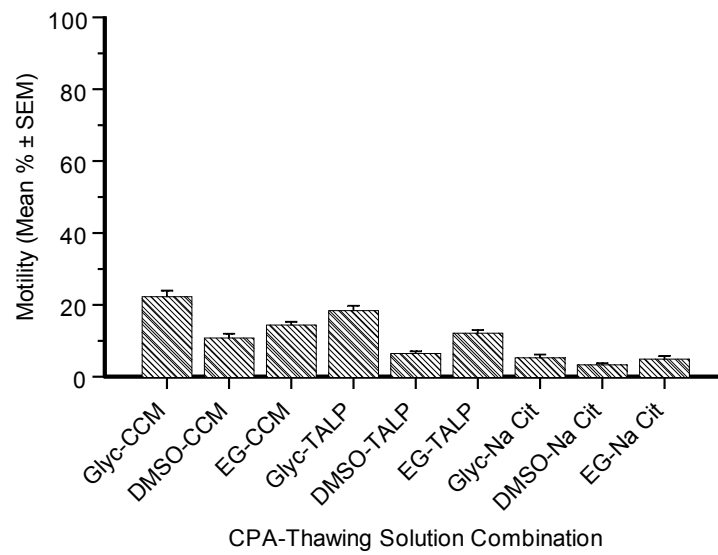
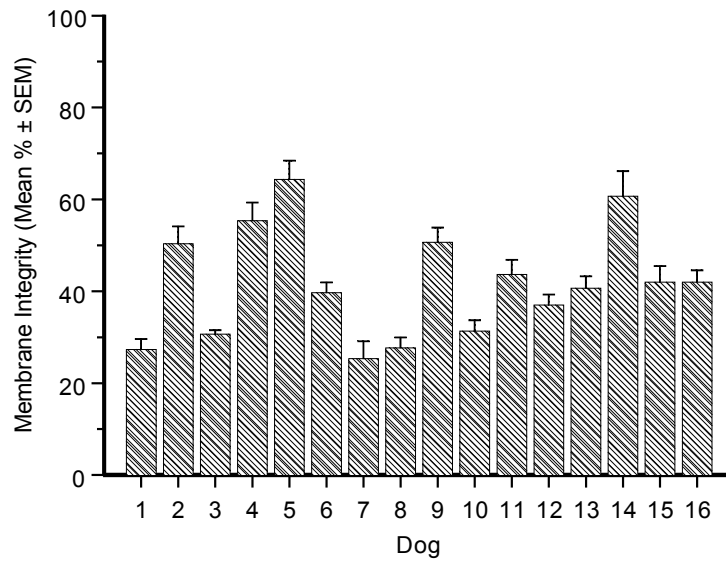


Figure 4.6. Mean survival of epididymal spermatozoa from 16 dogs as a function of CPA-thawing solution combinations. (A) Membrane Integrity. (B) Motility.

(A)



(B)

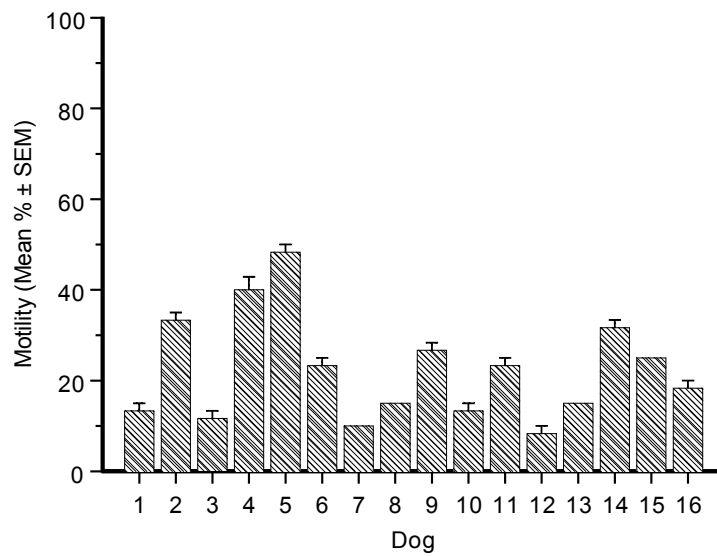


Figure 4.7. Survival of epididymal spermatozoa from individual dogs suspended in glycerol and thawed in CCM. (A) Membrane integrity. (B) Motility.

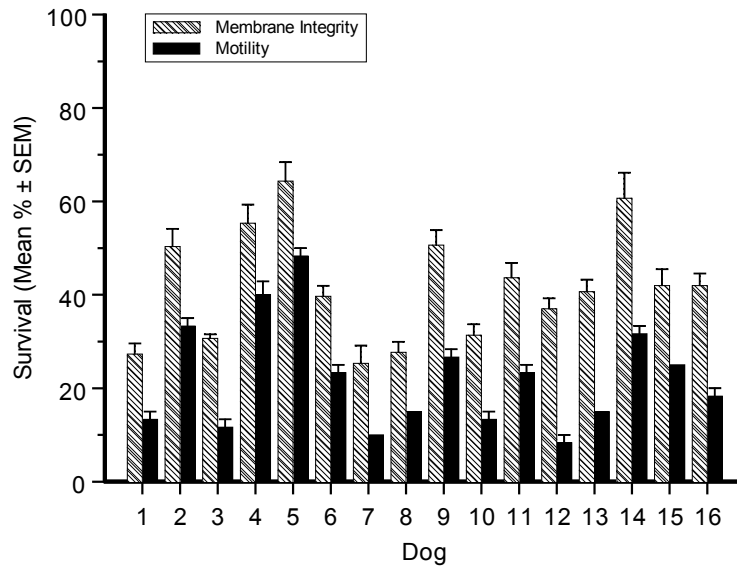


Figure 4.8. Mean values of membrane integrity and motility of epididymal spermatozoa from individual dogs treated suspended in glycerol and thawed in CCM.

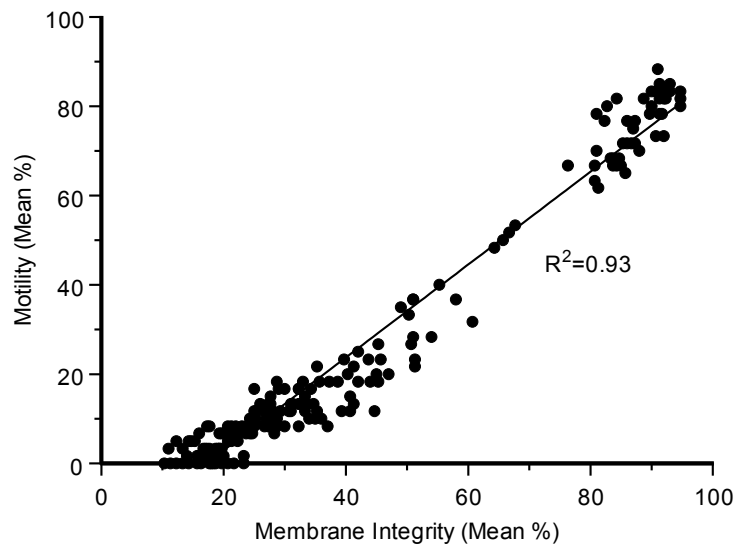


Figure 4.9. Mean motility of epididymal spermatozoa plotted as a function of mean membrane integrity of the same epididymal spermatozoa.

According to comparisons by analysis of variance, the age of the dog, the duration of time from surgery to the collection of spermatozoa, and the veterinary clinic from which the testes were obtained had no significant affect on the survival or the zona-binding capacity of the spermatozoa ($P>0.05$) (Appendix).

Experiment III

In this experiment, the capacity of epididymal spermatozoa from individual dogs to bind to canine zonae prior to pellet-freezing and after thawing was determined. Spermatozoa were suspended in 0.43 M glycerol, frozen as 100 μ l pellets, and thawed in CCM. The membrane integrity, motility, and number of membrane-intact sperm bound to each oocyte for replicate samples of pre-freeze and frozen-thawed spermatozoa are shown in Tables 4.6, 4.7, and 4.8, respectively. Tables 4.6.A and 4.6.B contain the same membrane integrity data, but, in Table 4.6.A, means are compared within each individual dog and, in Table 4.6.B, means are compared within each treatment group. Tables 4.7.A and 4.7.B contain the same motility data, but, in Table 4.7.A, means are compared within each individual dog and, in Table 4.7.B, means are compared within each treatment group. Tables 4.8.A and 4.8.B contain the number of membrane-intact sperm bound to each oocyte data, but, in Table 4.8.A, means are compared within each individual dog and, in Table 4.8.B, means are compared within each treatment group. Unfrozen samples held at room temperature had significantly lower survival than the fresh and cooled (pre-freeze control) samples, and the frozen-thawed samples had significantly lower survival than the unfrozen samples (Tables 4.6 and 4.7). The number of live sperm

Table 4.6. Effect of pellet-freezing and thawing on membrane integrity of epididymal spermatozoa.

A. Comparison of percentage membrane integrity within each individual dog.

Dog	Membrane Integrity (Mean % ± SEM)			
	Fresh	Pre-freeze	Unfrozen	Frozen
1	95.3 ± 0.9 ^a	93.7 ± 1.2 ^a	81.0 ± 2.4 ^a	53.5 ± 2.8 ^b
2	92.3 ± 2.3 ^a	89.3 ± 1.2 ^a	84.2 ± 2.3 ^a	46.2 ± 2.7 ^b
3	95.0 ± 2.6 ^a	91.0 ± 2.1 ^a	91.0 ± 1.6 ^a	72.7 ± 2.4 ^b
4	86.3 ± 4.4 ^a	85.7 ± 5.0 ^a	83.2 ± 3.6 ^a	44.3 ± 2.9 ^b
5	97.0 ± 1.5 ^a	96.0 ± 2.1 ^a	90.3 ± 2.6 ^a	44.3 ± 2.3 ^b
6	92.3 ± 2.7 ^{ab}	95.0 ± 2.3 ^a	74.7 ± 2.3 ^b	59.8 ± 3.0 ^c
7	87.3 ± 1.8 ^a	81.3 ± 2.9 ^a	70.8 ± 2.7 ^a	52.7 ± 3.2 ^b
8	91.0 ± 1.7 ^a	91.0 ± 2.1 ^a	90.2 ± 1.4 ^a	56.0 ± 2.6 ^b
9	96.3 ± 0.9 ^a	93.3 ± 1.5 ^a	88.2 ± 1.5 ^a	69.5 ± 3.6 ^a
10	91.7 ± 2.9 ^a	82.3 ± 2.6 ^a	83.7 ± 2.1 ^a	52.5 ± 3.6 ^b
11	95.7 ± 2.0 ^a	93.5 ± 3.2 ^a	93.0 ± 1.6 ^a	53.5 ± 2.8 ^b
12	96.7 ± 0.9 ^a	89.3 ± 3.8 ^a	83.7 ± 1.8 ^a	34.2 ± 3.3 ^b
13	92.7 ± 2.0 ^a	83.0 ± 4.7 ^a	83.5 ± 2.8 ^a	30.3 ± 3.4 ^b
14	94.0 ± 3.6 ^a	90.3 ± 3.3 ^a	83.2 ± 2.2 ^a	56.7 ± 2.0 ^b
15	87.7 ± 4.9 ^a	79.7 ± 2.7 ^a	81.2 ± 2.8 ^a	41.5 ± 3.8 ^b
MEAN	92.7 ± 0.8 ^a	89.0 ± 1.0 ^{ab}	84.1 ± 0.8 ^b	51.2 ± 1.4 ^c

Values with different superscripts within a row are significantly different (P<0.05).

B. Comparison of percentage membrane integrity within each treatment group.

Dog	Membrane Integrity (Mean % ± SEM)			
	Fresh	Pre-freeze	Unfrozen	Frozen
1	95.3 ± 0.9 ^a	93.7 ± 1.2 ^a	81.0 ± 2.4 ^{abc}	53.5 ± 2.8 ^{cde}
2	92.3 ± 2.3 ^a	89.3 ± 1.2 ^a	84.2 ± 2.3 ^{abc}	46.2 ± 2.7 ^{cdef}
3	95.0 ± 2.6 ^a	91.0 ± 2.1 ^a	91.0 ± 1.6 ^a	72.7 ± 2.4 ^a
4	86.3 ± 4.4 ^a	85.7 ± 5.0 ^a	83.2 ± 3.6 ^{abc}	44.3 ± 2.9 ^{defg}
5	97.0 ± 1.5 ^a	96.0 ± 2.1 ^a	90.3 ± 2.6 ^a	44.3 ± 2.3 ^{defg}
6	92.3 ± 2.7 ^a	95.0 ± 2.3 ^a	74.7 ± 2.3 ^{bc}	59.8 ± 3.0 ^{bcd}
7	87.3 ± 1.8 ^a	81.3 ± 2.9 ^a	70.8 ± 2.7 ^c	52.7 ± 3.2 ^{cde}
8	91.0 ± 1.7 ^a	91.0 ± 2.1 ^a	90.2 ± 1.4 ^a	56.0 ± 2.6 ^{bcde}
9	96.3 ± 0.9 ^a	93.3 ± 1.5 ^a	88.2 ± 1.5 ^{ab}	69.5 ± 3.6 ^{ab}
10	91.7 ± 2.9 ^a	82.3 ± 2.6 ^a	83.7 ± 2.1 ^{abc}	52.5 ± 3.6 ^{cde}
11	95.7 ± 2.0 ^a	93.5 ± 3.2 ^a	93.0 ± 1.6 ^a	53.5 ± 2.8 ^{cde}
12	96.7 ± 0.9 ^a	89.3 ± 3.8 ^a	83.7 ± 1.8 ^{abc}	34.2 ± 3.3 ^{fg}
13	92.7 ± 2.0 ^a	83.0 ± 4.7 ^a	83.5 ± 2.8 ^{abc}	30.3 ± 3.4 ^g
14	94.0 ± 3.6 ^a	90.3 ± 3.3 ^a	83.2 ± 2.2 ^{abc}	56.7 ± 2.0 ^{bcd}
15	87.7 ± 4.9 ^a	79.7 ± 2.7 ^a	81.2 ± 2.8 ^{abc}	41.5 ± 3.8 ^{efg}

Values with different superscripts within a column are significantly different (P<0.05).

Table 4.7. Effect of pellet-freezing and thawing on motility of epididymal spermatozoa.

A. Comparison of percentage motility within each individual dog.

Dog	Motility (Mean % ± SEM)			
	Fresh	Pre-freeze	Unfrozen	Frozen
1	83.3 ± 1.7 ^a	81.7 ± 1.7 ^a	62.5 ± 1.1 ^b	30.0 ± 1.8 ^c
2	73.3 ± 1.7 ^a	71.2 ± 1.7 ^a	65.0 ± 1.8 ^a	21.7 ± 1.1 ^b
3	86.7 ± 1.7 ^a	83.3 ± 1.7 ^a	80.9 ± 1.5 ^a	50.0 ± 1.3 ^b
4	70.0 ± 2.9 ^a	60.0 ± 0.0 ^{ab}	57.5 ± 1.1 ^b	20.0 ± 1.3 ^c
5	93.3 ± 1.7 ^a	85.0 ± 2.9 ^{ab}	80.0 ± 1.3 ^b	24.2 ± 1.5 ^c
6	85.0 ± 2.9 ^a	83.3 ± 1.7 ^a	55.0 ± 2.6 ^b	35.0 ± 1.3 ^c
7	71.7 ± 1.7 ^a	66.7 ± 1.7 ^a	50.8 ± 1.5 ^b	29.2 ± 2.0 ^c
8	81.7 ± 1.7 ^a	76.7 ± 1.7 ^a	72.5 ± 1.1 ^a	39.2 ± 1.5 ^b
9	91.2 ± 1.7 ^a	86.7 ± 1.7 ^a	70.8 ± 0.8 ^b	50.8 ± 1.5 ^c
10	73.3 ± 1.7 ^a	60.0 ± 0.0 ^{ab}	65.8 ± 1.5 ^b	31.7 ± 1.7 ^c
11	88.3 ± 1.7 ^a	81.7 ± 1.7 ^a	81.7 ± 1.1 ^a	27.5 ± 2.1 ^b
12	88.3 ± 1.7 ^a	81.7 ± 1.7 ^a	69.2 ± 2.4 ^b	17.5 ± 1.1 ^c
13	81.7 ± 1.7 ^a	73.3 ± 1.7 ^{ab}	67.5 ± 1.7 ^b	17.5 ± 2.1 ^c
14	78.3 ± 1.7 ^a	80.0 ± 2.9 ^a	66.7 ± 1.1 ^b	28.3 ± 1.7 ^c
15	76.7 ± 1.7 ^a	66.7 ± 1.7 ^{ab}	60.0 ± 1.8 ^b	18.3 ± 1.7 ^c
MEAN	81.6 ± 1.2 ^a	75.9 ± 1.4 ^a	67.1 ± 1.0 ^b	29.4 ± 1.2 ^c

Values with different superscripts within a row are significantly different (P<0.05).

B. Comparison of percentage motility within each treatment group.

Dog	Motility (Mean % ± SEM)			
	Fresh	Pre-freeze	Unfrozen	Frozen
1	83.3 ± 1.7 ^{abcd}	81.7 ± 1.7 ^{abc}	62.5 ± 1.1 ^{defg}	30.0 ± 1.8 ^{cde}
2	73.3 ± 1.7 ^{cde}	71.2 ± 1.7 ^{cde}	65.0 ± 1.8 ^{cdef}	21.7 ± 1.1 ^{efg}
3	86.7 ± 1.7 ^{ab}	83.3 ± 1.7 ^{abc}	80.9 ± 1.5 ^{ab}	50.0 ± 1.3 ^a
4	70.0 ± 2.9 ^e	60.0 ± 0.0 ^e	57.5 ± 1.1 ^{fgh}	20.0 ± 1.3 ^{fg}
5	93.3 ± 1.7 ^a	85.0 ± 2.9 ^{ab}	80.0 ± 1.3 ^{ab}	24.2 ± 1.5 ^{defg}
6	85.0 ± 2.9 ^{abc}	83.3 ± 1.7 ^{abc}	55.0 ± 2.6 ^{gh}	35.0 ± 1.3 ^{bc}
7	71.7 ± 1.7 ^{de}	66.7 ± 1.7 ^{de}	50.8 ± 1.5 ^h	29.2 ± 2.0 ^{cde}
8	81.7 ± 1.7 ^{abcde}	76.7 ± 1.7 ^{abcd}	72.5 ± 1.1 ^{bc}	39.2 ± 1.5 ^b
9	91.2 ± 1.7 ^a	86.7 ± 1.7 ^a	70.8 ± 0.8 ^{cd}	50.8 ± 1.5 ^a
10	73.3 ± 1.7 ^{cde}	60.0 ± 0.0 ^e	65.8 ± 1.5 ^{cdef}	31.7 ± 1.7 ^{bcd}
11	88.3 ± 1.7 ^{ab}	81.7 ± 1.7 ^{abc}	81.7 ± 1.1 ^a	27.5 ± 2.1 ^{cdef}
12	88.3 ± 1.7 ^{ab}	81.7 ± 1.7 ^{abc}	69.2 ± 2.4 ^{cd}	17.5 ± 1.1 ^g
13	81.7 ± 1.7 ^{abcde}	73.3 ± 1.7 ^{bcd}	67.5 ± 1.7 ^{cde}	17.5 ± 2.1 ^g
14	78.3 ± 1.7 ^{bcde}	80.0 ± 2.9 ^{abc}	66.7 ± 1.1 ^{cde}	28.3 ± 1.7 ^{cdef}
15	76.7 ± 1.7 ^{bcde}	66.7 ± 1.7 ^{de}	60.0 ± 1.8 ^{efg}	18.3 ± 1.7 ^g

Values with different superscripts within a column are significantly different (P<0.05).

Table 4.8. Effect of pellet-freezing and thawing on number of membrane-intact epididymal spermatozoa bound per oocyte.

A. Comparison of number of membrane-intact spermatozoa bound per oocyte within each individual dog.

Dog	Sperm Bound/Oocyte (Mean # ± SEM)	
	Unfrozen	Frozen
1	60 ± 12 ^a	37 ± 9 ^a
2	45 ± 12 ^a	55 ± 9 ^a
3	36 ± 6 ^a	24 ± 4 ^a
4	126 ± 10 ^a	77 ± 10 ^b
5	60 ± 17 ^a	65 ± 10 ^a
6	65 ± 11 ^a	25 ± 4 ^a
7	14 ± 3 ^a	18 ± 4 ^a
8	12 ± 2 ^a	15 ± 2 ^a
9	6 ± 1 ^a	4 ± 1 ^a
10	57 ± 12 ^a	38 ± 5 ^a
11	130 ± 10 ^a	87 ± 11 ^a
12	29 ± 7 ^a	10 ± 2 ^a
13	74 ± 7 ^a	66 ± 8 ^a
14	67 ± 9 ^a	70 ± 11 ^a
15	22 ± 7 ^a	29 ± 8 ^a
MEAN	54 ± 4 ^a	41 ± 3 ^b

Values with different superscripts within a row are significantly different (P<0.05).

B. Comparison of number of membrane-intact spermatozoa bound per oocyte within each treatment group.

Dog	Sperm Bound/Oocyte (Mean # ± SEM)	
	Unfrozen	Frozen
1	60 ± 12 ^{bcd}	37 ± 9 ^{bcd}
2	45 ± 12 ^{bcd}	55 ± 9 ^{bcd}
3	36 ± 6 ^{bcd}	24 ± 4 ^{def}
4	126 ± 10 ^a	77 ± 10 ^{ab}
5	60 ± 17 ^{bcd}	65 ± 10 ^{abcd}
6	65 ± 11 ^{bcd}	25 ± 4 ^{def}
7	14 ± 3 ^{ef}	18 ± 4 ^{ef}
8	12 ± 2 ^f	15 ± 2 ^{ef}
9	6 ± 1 ^f	4 ± 1 ^f
10	57 ± 12 ^{bcd}	38 ± 5 ^{bcd}
11	130 ± 10 ^a	87 ± 11 ^a
12	29 ± 7 ^{cdef}	10 ± 2 ^f
13	74 ± 7 ^b	66 ± 8 ^{abcd}
14	67 ± 9 ^{bc}	70 ± 11 ^{abc}
15	22 ± 7 ^{def}	29 ± 8 ^{cdef}

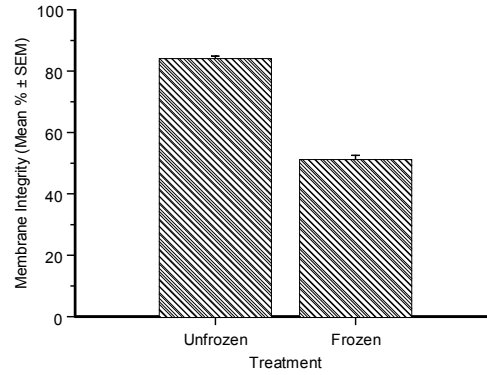
Values with different superscripts within a column are significantly different (P<0.05).

bound to each oocyte was significantly higher in unfrozen samples than in frozen-thawed samples (Table 4.8). The mean membrane integrity for frozen-thawed spermatozoa was 51%, and the mean motility was 29% (Figures 4.10.A and 4.10.B). The corresponding values from Experiment I were 32% and 43%, respectively. For Experiment II, the mean values for the same treatments were 22% and 42%, respectively. The mean number of frozen-thawed membrane-intact spermatozoa bound to the zona of an individual oocyte was 41 (Figure 4.10.C).

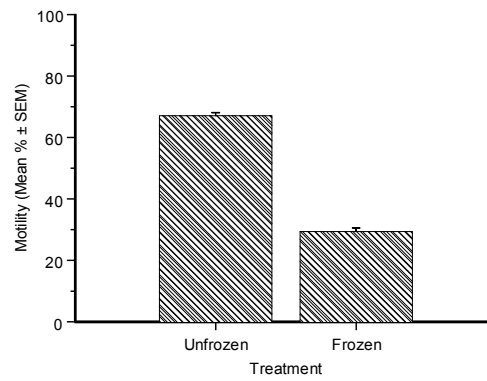
Because epididymal spermatozoa were used for these experiments, it was only possible to make single observations for each dog with duplicate samples subjected to each treatment. Comparisons of survival of spermatozoa were made from individual dogs treated separately. For frozen-thawed spermatozoa, mean percentages of membrane integrity ranged from 30% for Dog 13 to 73% for Dog 3 (Figure 4.11.A), and there were significant differences among individual dogs (Table 4.6). The mean motilities varied significantly among individual dogs (Table 4.7), ranging from 18% for Dogs 12, 13, and 18 to 51% for Dog 9 (Figure 4.11.B). The mean number of membrane-intact spermatozoa bound to zonae was highly variable for both fresh and frozen spermatozoa (Table 4.8). For the former, it ranged from 6 spermatozoa/oocyte for Dog 9 to 130 spermatozoa /oocyte for Dog 11. For frozen samples, it ranged from 4 sperm/oocyte for Dog 9 to 87 sperm/oocyte for Dog 11 (Figure 4.11.C).

The results in Figure 4.12 show the mean values of membrane integrity and motility for each dog. For all dogs, the mean value of membrane integrity

(A)



(B)



(C)

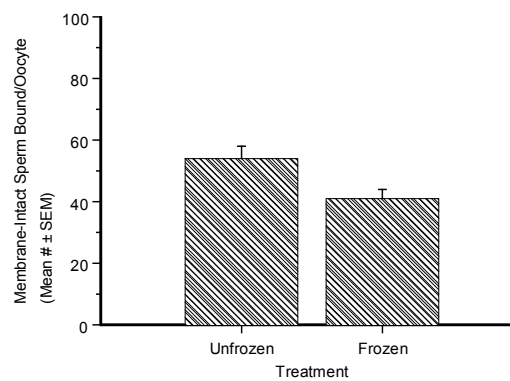
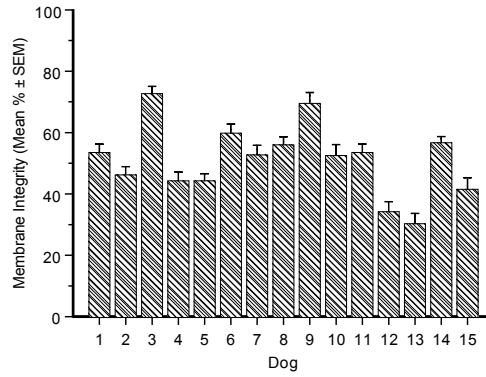
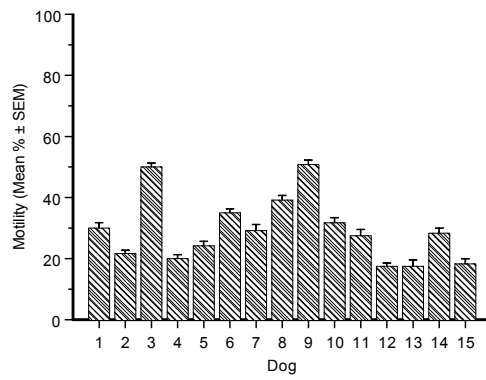


Figure 4.10. Mean value of survival of epididymal spermatozoa as a function of treatment group. (A) Membrane integrity. (B) Motility. (C) Membrane-intact sperm bound per oocyte.

(A)



(B)



(C)

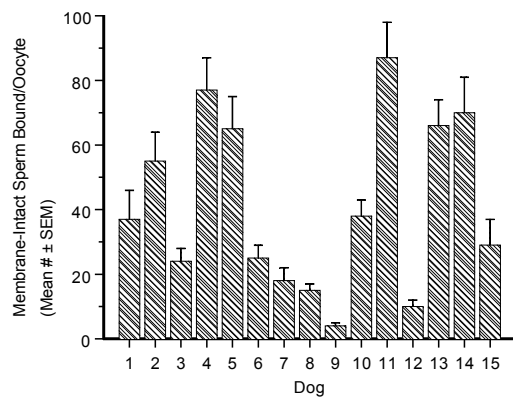


Figure 4.11. Survival of epididymal spermatozoa from individual dogs suspended in glycerol, cooled as 100 μ l pellets, and thawed in CCM. (A) Membrane Integrity. (B) Motility. (C) Membrane-intact sperm bound per oocyte.

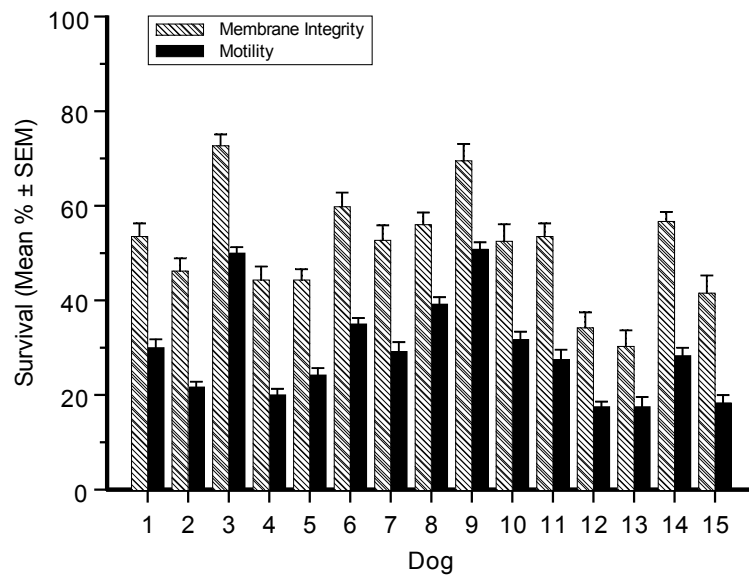


Figure 4.12. Mean values of membrane integrity and motility for epididymal spermatozoa of individual dogs suspended in glycerol, cooled as 100 μ l pellets, and thawed in CCM.

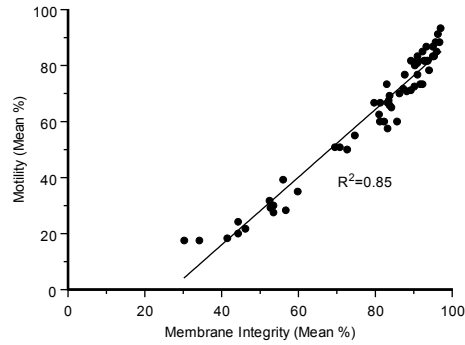
was greater than the mean value for motility. This was true for spermatozoa from dogs 2, 4, 14, and 15. There was a linear correlation between the membrane integrity and motility of all samples of spermatozoa (Figure 4.13.A). In contrast, there was not a correlation between membrane integrity and number of live sperm bound to each oocyte or between motility and number of live sperm bound to each oocyte (Figures 4.13.B and 4.13.C).

When an analysis of variance was performed, the age of the dog, the duration of time from surgery to the collection of spermatozoa, and the veterinary clinic from which the testes were obtained had no significant affect on the survival or the zona-binding capacity of the spermatozoa ($P>0.05$) (Appendix).

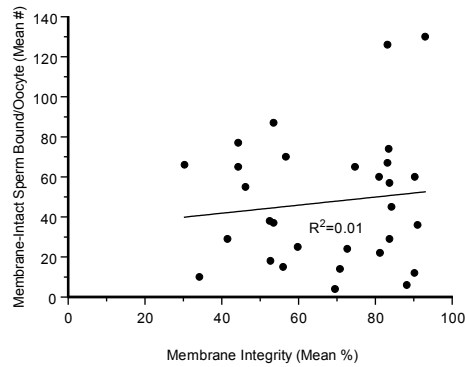
Experiment IV

In this experiment, ejaculated spermatozoa were cryopreserved by pellet-freezing to compare the post-thaw survival of ejaculated spermatozoa from three dogs. For comparison of these data to those in Experiments I, II, and III, I also froze additional samples of epididymal spermatozoa and to determine the reproducibility of the post-thaw survival of spermatozoa for a given dog. Spermatozoa were suspended in 0.43 M glycerol, frozen as 100 μ l pellets, and thawed in CCM. For the ejaculated spermatozoa, three observations were obtained for each of three dogs by collecting three ejaculates from each of the dogs, and each observation was based on duplicate samples subjected to each treatment. Comparisons of survival of spermatozoa were made for individual dogs treated separately. For each dog from which ejaculates were collected, there was only slight variation in survival among individual ejaculates (Table 4.9;

(A)



(B)



(C)

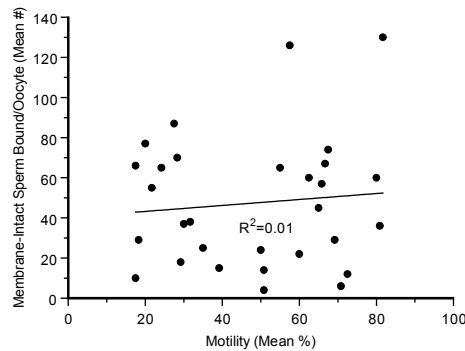


Figure 4.13. Linear correlations of survival of epididymal spermatozoa. (A) Motility of epididymal spermatozoa as a function of membrane integrity of the same spermatozoa. (B) Membrane-intact epididymal spermatozoa bound per oocyte as a function of membrane integrity of the same spermatozoa. (C) Membrane-intact epididymal spermatozoa bound per oocyte of epididymal spermatozoa as a function of motility of the same spermatozoa.

Table 4.9. Survival of spermatozoa from individual ejaculates of individual dogs.

A. Comparison of survival for each dog by ejaculate for Unfrozen treatment.

Dog	Assay	Ejaculate 1	Ejaculate 2	Ejaculate 3	Mean
1	Membrane Integrity	91.5 ± 1.4 ^a	94.2 ± 1.2 ^a	93.7 ± 1.5 ^a	93.1 ± 0.8
	Motility	75.8 ± 1.5 ^b	84.2 ± 0.8 ^a	89.2 ± 0.8 ^a	83.1 ± 1.5
	Sperm Bound/Oocyte	62 ± 9 ^a	78 ± 8 ^a	87 ± 8 ^a	76 ± 5
2	Membrane Integrity	89.5 ± 1.7 ^a	93.0 ± 1.9 ^a	90.0 ± 1.4 ^a	90.8 ± 1.0
	Motility	69.2 ± 1.5 ^b	69.2 ± 0.8 ^b	78.3 ± 1.1 ^a	72.2 ± 1.2
	Sperm Bound/Oocyte	36 ± 7 ^a	37 ± 4 ^a	36 ± 4 ^a	37 ± 4
3	Membrane Integrity	80.0 ± 1.2 ^b	93.5 ± 1.7 ^a	92.8 ± 1.1 ^a	88.8 ± 1.4
	Motility	55.8 ± 1.5 ^b	79.2 ± 1.5 ^a	85.8 ± 0.8 ^a	73.6 ± 3.2
	Sperm Bound/Oocyte	48 ± 5 ^a	48 ± 7 ^a	60 ± 6 ^a	52 ± 4

Value with different superscripts within a row are significantly different (P<0.05).

B. Comparison of survival for each dog by ejaculate for Frozen treatment.

Dog	Assay	Ejaculate 1	Ejaculate 2	Ejaculate 3	Mean
1	Membrane Integrity	59.7 ± 1.6 ^a	47.7 ± 2.7 ^b	61.2 ± 1.4 ^a	56.2 ± 1.8
	Motility	35.0 ± 1.3 ^{ab}	28.3 ± 1.3 ^b	40.0 ± 1.7 ^a	34.4 ± 1.4
	Sperm Bound/Oocyte	70 ± 8 ^a	61 ± 7 ^a	71 ± 9 ^a	68 ± 5
2	Membrane Integrity	41.5 ± 2.2 ^a	31.7 ± 1.9 ^b	43.8 ± 2.7 ^a	39.0 ± 1.8
	Motility	17.5 ± 1.1 ^{ab}	12.5 ± 1.1 ^b	20.0 ± 1.3 ^a	16.7 ± 1.0
	Sperm Bound/Oocyte	31 ± 7 ^a	21 ± 7 ^a	28 ± 4 ^a	27 ± 3
3	Membrane Integrity	33.8 ± 2.1 ^a	35.5 ± 2.3 ^a	41.8 ± 1.4 ^a	37.1 ± 1.4
	Motility	15.0 ± 1.3 ^a	15.8 ± 1.5 ^a	21.7 ± 1.7 ^a	17.5 ± 1.1
	Sperm Bound/Oocyte	49 ± 7 ^a	45 ± 7 ^a	46 ± 7 ^a	47 ± 4

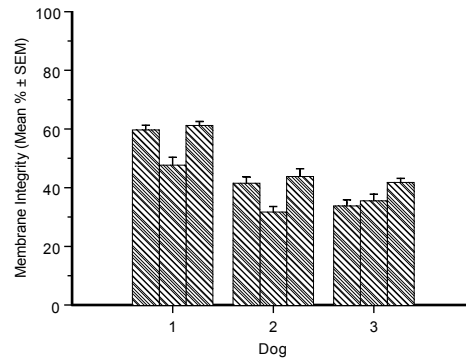
Value with different superscripts within a row are significantly different (P<0.05).

Figure 4.14), which supports the assumption that the values of post-thaw survival of spermatozoa from an individual dog are reproducible.

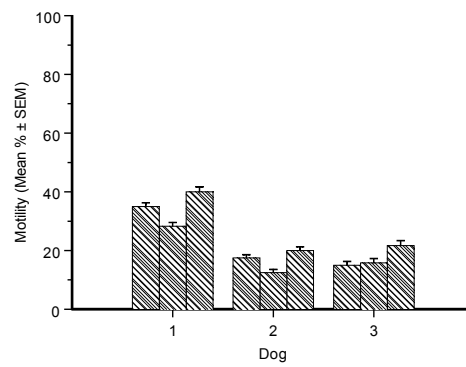
The membrane integrity, motility, and number of membrane-intact sperm bound to each oocyte for replicate samples of ejaculated and epididymal spermatozoa are shown in Tables 4.10, 4.11, and 4.12. Tables 4.10.A and 4.10.B contain the same membrane integrity data, but, in Table 4.10.A, means are compared within each individual dog and, in Table 4.10.B, means are compared within each treatment group. Tables 4.11.A and 4.11.B contain the same motility data, but, in Table 4.11.A, means are compared within each individual dog and, in Table 4.11.B, means are compared within each treatment group. Tables 4.12.A and 4.12.B show the data for the number of membrane-intact sperm bound to each oocyte, but, in Table 4.12.A, the means are compared within each dog, and, in Table 4.12.B, the means are compared within each treatment group. The frozen-thawed samples had significantly lower survival than the unfrozen samples (Tables 4.10 and 4.11). The number of membrane-intact sperm bound to each oocyte was significantly higher in unfrozen samples than in frozen-thawed samples (Table 4.12).

The mean values of motility and membrane integrity of epididymal and ejaculated spermatozoa are shown in Table 4.13. The mean membrane integrity for frozen-thawed ejaculated spermatozoa was 44%, and the mean motility was 23%. The mean number of frozen-thawed membrane-intact ejaculated spermatozoa bound to the zona of an individual oocyte was 47. The mean motility for frozen-thawed

(A)



(B)



(C)

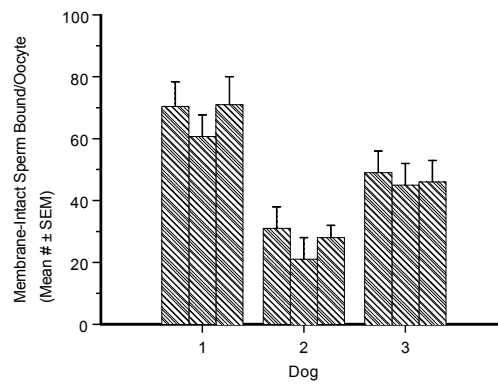


Figure 4.14. Post-thaw survival of spermatozoa from individual ejaculates from individual dogs. (A) Membrane integrity. (B) Motility. (C) Membrane-intact sperm bound per oocyte.

Table 4.10. Effect of pellet-freezing and thawing on membrane integrity of ejaculated and epididymal spermatozoa.

A. Comparison of percentage membrane integrity from each dog.

Sperm Category	Dog	Membrane Integrity (Mean % \pm SEM)			
		Fresh	Pre-freeze	Unfrozen	Frozen
Ejaculated	1	95.4 \pm 1.1 ^a	94.4 \pm 1.3 ^a	93.1 \pm 0.8 ^a	56.2 \pm 1.8 ^b
	2	92.8 \pm 1.4 ^a	91.0 \pm 1.2 ^a	90.8 \pm 1.0 ^a	39.0 \pm 1.8 ^b
	3	95.2 \pm 1.1 ^a	92.4 \pm 0.7 ^a	88.8 \pm 1.7 ^a	37.1 \pm 1.4 ^b
Epididymal	4	93.7 \pm 1.2 ^a	88.0 \pm 2.1 ^a	87.8 \pm 1.4 ^a	47.8 \pm 2.9 ^b
	5	96.3 \pm 1.2 ^a	96.0 \pm 1.7 ^a	94.3 \pm 1.1 ^a	41.2 \pm 1.9 ^b
	6	94.3 \pm 0.9 ^a	92.3 \pm 1.7 ^a	92.0 \pm 1.4 ^a	60.3 \pm 2.8 ^b
	7	96.3 \pm 1.2 ^a	95.0 \pm 1.5 ^a	92.3 \pm 1.2 ^a	31.7 \pm 1.4 ^b

Values with different superscripts within a row are significantly different (P<0.05).

B. Comparison of percentage membrane integrity within each treatment group.

Sperm Category	Dog	Membrane Integrity (Mean % \pm SEM)			
		Fresh	Pre-freeze	Unfrozen	Frozen
Ejaculated	1	95.4 \pm 1.1 ^a	94.4 \pm 1.3 ^a	93.1 \pm 0.8 ^a	56.2 \pm 1.8 ^{ab}
	2	92.8 \pm 1.4 ^a	91.0 \pm 1.2 ^a	90.8 \pm 1.0 ^a	39.0 \pm 1.8 ^{cd}
	3	95.2 \pm 1.1 ^a	92.4 \pm 0.7 ^a	88.8 \pm 1.7 ^a	37.1 \pm 1.4 ^d
Epididymal	4	93.7 \pm 1.2 ^a	88.0 \pm 2.1 ^a	87.8 \pm 1.4 ^a	47.8 \pm 2.9 ^{bc}
	5	96.3 \pm 1.2 ^a	96.0 \pm 1.7 ^a	94.3 \pm 1.1 ^a	41.2 \pm 1.9 ^{cd}
	6	94.3 \pm 0.9 ^a	92.3 \pm 1.7 ^a	92.0 \pm 1.4 ^a	60.3 \pm 2.8 ^a
	7	96.3 \pm 1.2 ^a	95.0 \pm 1.5 ^a	92.3 \pm 1.2 ^a	31.7 \pm 1.4 ^d

Values with different superscripts within a column are significantly different (P<0.05).

Table 4.11. Effect of pellet-freezing and thawing on motility of ejaculated and epididymal spermatozoa.

A. Comparison of percentage motility of spermatozoa from each dog.

Sperm Category	Dog	Motility (Mean % \pm SEM)			
		Fresh	Pre-freeze	Unfrozen	Frozen
Ejaculated	1	89.4 \pm 1.0 ^a	83.3 \pm 0.8 ^a	83.1 \pm 1.5 ^a	34.4 \pm 1.4 ^b
	2	76.1 \pm 1.6 ^a	71.1 \pm 1.4 ^a	72.2 \pm 1.2 ^a	16.7 \pm 1.0 ^b
	3	85.0 \pm 1.2 ^a	76.1 \pm 2.7 ^{ab}	73.6 \pm 3.2 ^b	17.5 \pm 1.1 ^c
Epididymal	4	81.7 \pm 1.7 ^a	66.7 \pm 1.7 ^{ab}	64.2 \pm 2.0 ^b	25.8 \pm 1.5 ^c
	5	88.3 \pm 1.7 ^a	80.0 \pm 2.9 ^a	81.7 \pm 1.1 ^a	20.0 \pm 1.3 ^b
	6	81.7 \pm 1.7 ^a	68.3 \pm 1.7 ^a	75.8 \pm 0.8 ^a	39.2 \pm 1.5 ^b
	7	90.0 \pm 0.0 ^a	83.3 \pm 1.7 ^a	85.8 \pm 1.5 ^a	14.2 \pm 0.9 ^b

Values with different superscripts within a row are significantly different (P<0.05).

B. Comparison of percentage motility of spermatozoa within each treatment group.

Sperm Category	Dog	Motility (Mean % \pm SEM)			
		Fresh	Pre-freeze	Unfrozen	Frozen
Ejaculated	1	89.4 \pm 1.0 ^{ab}	83.3 \pm 0.8 ^a	83.1 \pm 1.5 ^a	34.4 \pm 1.4 ^{ab}
	2	76.1 \pm 1.6 ^b	71.1 \pm 1.4 ^b	72.2 \pm 1.2 ^{bc}	16.7 \pm 1.0 ^c
	3	85.0 \pm 1.2 ^{ab}	76.1 \pm 2.7 ^{ab}	73.6 \pm 3.2 ^{bc}	17.5 \pm 1.1 ^c
Epididymal	4	81.7 \pm 1.7 ^{ab}	66.7 \pm 1.7 ^b	64.2 \pm 2.0 ^c	25.8 \pm 1.5 ^{bc}
	5	88.3 \pm 1.7 ^{ab}	80.0 \pm 2.9 ^{ab}	81.7 \pm 1.1 ^{ab}	20.0 \pm 1.3 ^c
	6	81.7 \pm 1.7 ^{ab}	68.3 \pm 1.7 ^b	75.8 \pm 0.8 ^{abc}	39.2 \pm 1.5 ^a
	7	90.0 \pm 0.0 ^a	83.3 \pm 1.7 ^a	85.8 \pm 1.5 ^a	14.2 \pm 0.9 ^c

Values with different superscripts within a column are significantly different (P<0.05).

Table 4.12. Effect of pellet-freezing and thawing on membrane-intact ejaculated and epididymal spermatozoa bound per oocyte.

A. Comparison of number of membrane-intact sperm bound per oocyte for each dog.

Sperm Category	Dog	Sperm Bound/Oocyte (Mean # ± SEM)	
		Unfrozen	Frozen
Ejaculated	1	76 ± 5 ^a	68 ± 5 ^a
	2	37 ± 4 ^a	27 ± 3 ^a
	3	52 ± 4 ^a	47 ± 4 ^a
Epididymal	4	72 ± 8 ^a	45 ± 6 ^a
	5	66 ± 7 ^a	46 ± 7 ^a
	6	60 ± 9 ^a	37 ± 8 ^a
	7	22 ± 4 ^a	10 ± 2 ^a

Values with different superscripts within a row are significantly different (P<0.05).

B. Comparison of number of membrane-intact sperm bound per oocyte within each treatment group.

Sperm Category	Dog	Sperm Bound/Oocyte (Mean # ± SEM)	
		Unfrozen	Frozen
Ejaculated	1	76 ± 5 ^a	68 ± 5 ^a
	2	37 ± 4 ^{cd}	27 ± 3 ^{cd}
	3	52 ± 4 ^{bc}	47 ± 4 ^b
Epididymal	4	72 ± 8 ^{ab}	45 ± 6 ^{abc}
	5	66 ± 7 ^{ab}	46 ± 7 ^{abc}
	6	60 ± 9 ^{abc}	37 ± 8 ^{bcd}
	7	22 ± 4 ^d	10 ± 2 ^d

Values with different superscripts within a column are significantly different (P<0.05).

Table 4.13. Mean survival of ejaculated and epididymal spermatozoa by sperm category.

Treatment Group	Sperm Category	Motility (Mean % ± SEM)	Membrane Integrity (Mean % ± SEM)	Membrane-Intact Sperm Bound per oocyte (Mean # ± SEM)
Unfrozen	Ejaculated	76.3 ± 1.8 ^a	90.9 ± 2.6 ^a	55 ± 6 ^a
	Epididymal	76.9 ± 1.7 ^a	92.4 ± 2.1 ^a	55 ± 9 ^a
Frozen	Ejaculated	22.9 ± 2.3 ^a	44.1 ± 1.2 ^a	47 ± 7 ^a
	Epididymal	24.8 ± 2.1 ^a	35.3 ± 1.1 ^a	34 ± 5 ^b

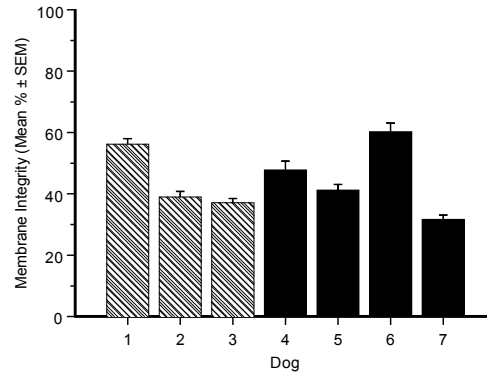
Values with different superscripts within a column within a treatment group are significantly different (P<0.05).

epididymal spermatozoa was 25%, and the mean membrane integrity was 35%. The mean number of frozen-thawed membrane-intact epididymal spermatozoa bound to the zona of an individual oocyte was 34. The mean values of motility and membrane integrity of frozen-thawed epididymal and ejaculated spermatozoa were statistically the same while the mean numbers of membrane-intact sperm bound to the zona of each oocyte were significantly different (Table 4.13).

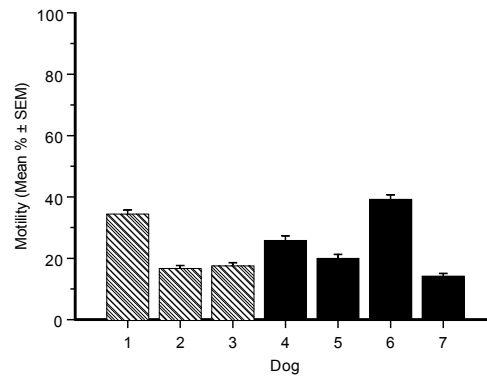
For frozen-thawed ejaculated spermatozoa, membrane integrity was significantly different among dogs (Table 4.10), ranging from 37% for Dog 3 to 56% for Dog 1 (Figure 4.15.A). Motility ranged from 17% for Dog 2 to 34% for Dog 1 (Figure 4.15.B), and there were significant differences among dogs (Table 4.11). The number of membrane-intact sperm bound to the zona of each oocyte varied significantly (Table 4.12), from 27 for Dog 2 to 68 for Dog 1 (Figure 4.15.C).

For the epididymal spermatozoa, it was only possible to make single observations for each dog with duplicate samples subjected to each treatment. Comparisons of survival of spermatozoa were made for individual dogs treated separately. There were significant differences among the values of membrane integrity of spermatozoa from individual dogs (Table 4.10), ranging from 32% for Dog 7 to 60% for Dog 6 (Figure 4.15.A). Motility was significantly different among dogs (Table 4.11) and ranged from 14% for Dog 7 to 39 for Dog 6 (Figure 4.15.B). The number of membrane-intact sperm bound to the zona of each

(A)



(B)



(C)

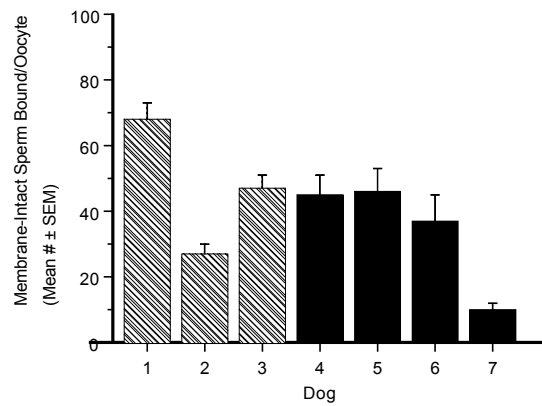


Figure 4.15. Survival of spermatozoa from individual dogs. Striped bars represent ejaculated samples, and solid bars represent epididymal samples (A) Membrane Integrity. (B) Motility. (C) Membrane-intact sperm bound per oocyte.

oocyte were variable and were significantly different (Table 4.12), from 10 for Dog 7 to 45 for Dog 4 (Figure 4.15.C).

The results in Figure 4.16 show the mean values of membrane integrity and motility for each dog. For both ejaculated and epididymal spermatozoa from each dog, the value for membrane integrity was greater than the value for motility. This was especially true for the ejaculated spermatozoa from dogs 2 and 3 and for the epididymal spermatozoa from dogs 5 and 7. As shown in Figure 4.17.A, there was a linear correlation between the membrane integrity and motility of all samples of spermatozoa. In contrast, there was not a correlation between membrane integrity and number of live sperm bound to each oocyte or between motility and number of live sperm bound to each oocyte (Figures 4.17.B and 4.17.C).

Based on comparisons by analysis of variance, the age of the dog, the duration of time from surgery to the collection of spermatozoa, and the veterinary clinic from which the testes were obtained had no significant affect on the survival or the zona-binding capacity of the spermatozoa ($P>0.5$) (Appendix).

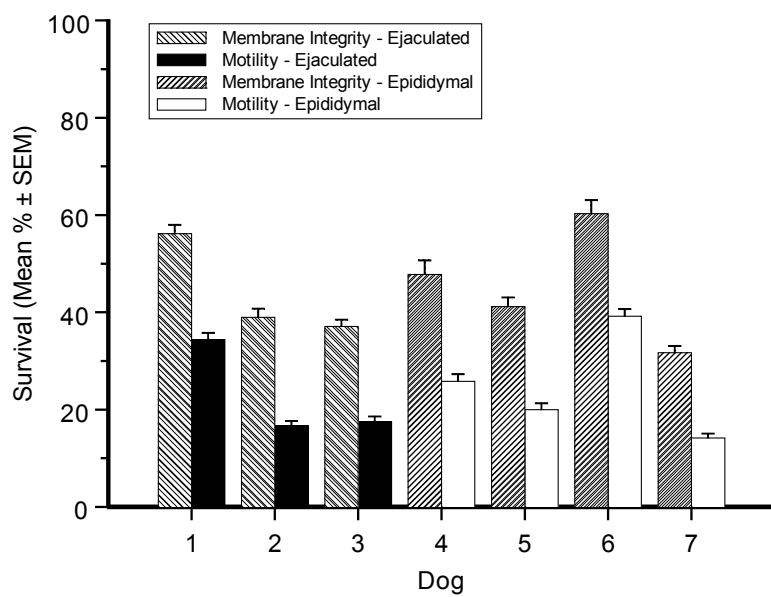
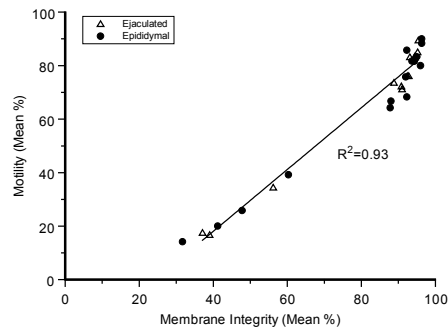
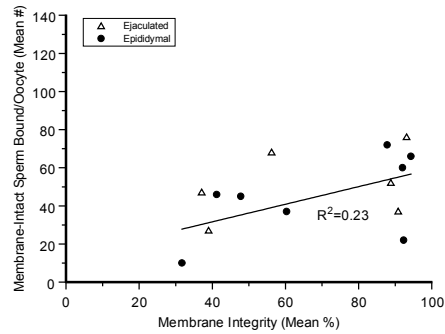


Figure 4.16. Mean values of membrane integrity and motility of ejaculated and epididymal spermatozoa from individual dogs suspended in glycerol, cooled as pellets of 100 μ l, and thawed in CCM.

(A)



(B)



(C)

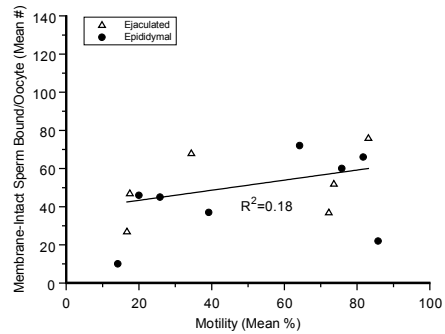


Figure 4.17. Linear correlations of survival of ejaculated and epididymal spermatozoa. (A) Motility of ejaculated and epididymal spermatozoa plotted as a function of membrane integrity of the same ejaculated and epididymal spermatozoa. (B) Membrane-intact ejaculated and epididymal spermatozoa bound per oocyte plotted as a function of membrane integrity of the same ejaculated and epididymal spermatozoa. (C) Membrane-intact ejaculated and epididymal spermatozoa bound per oocyte of ejaculated and epididymal spermatozoa plotted as a function of motility of the same epididymal spermatozoa.

CHAPTER V

DISCUSSION

The four experiments described in this thesis were designed to examine variables of the pellet method for freezing canine spermatozoa. Although this method was first described by Seager (1969) more than 30 years ago, it has not been recently studied in detail. The overall purposes of this study were (1) to determine the effects of pellet volumes, cryoprotective agents, and thawing solutions on post-thaw survival of epididymal spermatozoa from individual dogs and (2) to determine the zona-binding capacity of epididymal spermatozoa from individual dogs prior to pellet-freezing and after thawing. Using the method found to produce the greatest survival for epididymal spermatozoa, canine ejaculated spermatozoa was cryopreserved to compare post-thaw survival and zona-binding capacity of the two categories of spermatozoa and to determine the reproducibility of the post-thaw survival of spermatozoa within an individual dog.

The results of Experiment I indicate that canine epididymal spermatozoa exhibit maximum survival when frozen as pellets of 100 μ l volume on dry ice (Figure 4.1). However, post-thaw sperm survival varies among dogs, and the pellet volume that yields maximum survival is variable for individual dogs.

The data also suggest that the pellet volume affects cooling rate, which has been found to affect sperm survival (Yu et al., 2002). When membrane integrity and motility were plotted as a function of pellet volume, an inverted V-shaped curve was the result (Figure 4.1). The results suggest that the two-factor hypothesis of freezing injury (Mazur et al., 1972) can explain the survival of canine epididymal spermatozoa even when frozen as pellets. At low cooling

rates, one factor is hypothesized to damage cells by exposing them to solutions that have become more concentrated by the removal of water in the form of ice. Damage is hypothesized to occur because slow cooling is equivalent to extended exposure to altered solutions. At higher cooling rates, a second factor is considered to result from intracellular ice formation. High cooling rates mean that there is insufficient time for water to flow out of cells, which become increasingly supercooled and more likely to undergo intracellular nucleation at low subzero temperatures. The results reported herein of the effect of pellet volume, and thus cooling rate, are similar to those reported for spermatozoa of boars, rams, bulls, and humans (Duncan and Watson, 1992; Fiser et al., 1993; Henry et al., 1993; Woelders et al., 1997), as well as those of epididymal spermatozoa of dogs (Yu et al., 2002).

The results of Experiment II suggest that canine epididymal spermatozoa exhibit their overall maximum survival when treated with the CPA glycerol and thawed in CCM or TALP medium (Figure 4.6). However, post-thaw sperm survival varies among dogs, and the CPA-thawing solution combination that yields maximum survival for individual dogs does vary.

The CPAs used in this experiment, glycerol, dimethyl sulfoxide, and ethylene glycol, permeate cells and provide protection to the cell from within. For spermatozoa of most species, glycerol has been the most commonly used cryoprotectant, but dimethyl sulfoxide has sometimes been used alone or in combination with glycerol (England, 1993). The optimal glycerol concentration varies with diluent, cooling method, and species. The optimum cryoprotectant concentration depends on cooling rate as well; lower concentrations require faster freezing rates. In the dog, glycerol concentrations that have been used

successfully vary between 4 and 11% (v/v) depending on diluent composition (England, 1993).

In the present experiments, the use of glycerol as a CPA resulted in the highest overall survival of canine epididymal spermatozoa when compared to dimethyl sulfoxide and ethylene glycol. In these experiments, a final CPA concentration of 0.43 M was used because it is approximately equal to 4% (v/v). Seager (1969) indicated that 4% (v/v) was the glycerol concentration that resulted in maximum survival of spermatozoa after thawing. A glycerol concentration of approximately 4% (v/v) also resulted in maximum survival in several other studies of the freezing of canine epididymal spermatozoa (Fontbonne and Badinand, 1993; Hewitt et al., 2001; Olar et al., 1989; Rota et al., 1998). Because glycerol has been so successfully used as a CPA for canine spermatozoa, there have been few studies of the cryopreservation of canine spermatozoa using dimethyl sulfoxide or ethylene glycol as a CPA.

In these experiments, the frozen pellets of spermatozoa were thawed and diluted in sodium citrate, TALP, or CCM. Usually, pelleted semen is thawed in a solution of saline or sodium citrate at 37°C (Linde-Forsberg, 1991). Other solutions, such as TALP and CCM, have also been used to dilute spermatozoa after freezing and thawing (Mahi and Yanagimachi, 1978; Yu et al., 2002). Mahi and Yanagimachi (1978) reported that CCM was effective in supporting both the motility and the acrosome reaction of canine spermatozoa. In these experiments, thawing and dilution of canine epididymal spermatozoa in CCM resulted in the highest overall post-thaw survival, and CCM supported the motility of canine epididymal spermatozoa more effectively compared to sodium citrate and TALP.

A zona-binding assay was used in these experiments to estimate fertilizing potential of frozen-thawed canine epididymal spermatozoa. Assayed this way, epididymal spermatozoa from individual dogs decreased after pellet-freezing and thawing (Figure 4.10). Although the zona-binding capacity varied greatly among dogs and did not correlate with motility or membrane integrity in this experiment, survival of frozen-thawed spermatozoa as determined by all three assays decreased overall compared to unfrozen spermatozoa (Figure 4.10). Hay et al. (1997b) had also studied the effects of cryopreservation on penetration of canine oocytes by spermatozoa, and they found that although sperm cells seemed to survive freezing, as determined by their membrane integrity and motility, there was a marked decrease in the number of spermatozoa capable of penetrating homologous oocytes. Hay et al. (1997a) also showed that decreased motility and increased acrosomal damage to canine sperm cells was correlated with reduced penetration of homologous oocytes after freezing and thawing.

There was little variation in survival among ejaculates from individual dogs (Figure 4.14); this supports the assumption that the values for post-thaw survival of spermatozoa from an individual dog are reproducible. These results substantiate the validity of the results of Experiments I, II, and III, in which epididymal spermatozoa from an individual dog could only be sampled once. That is, the reproducibility of values for ejaculated spermatozoa indicate that the single determinations of epididymal spermatozoa are valid. The mean values of motility and membrane integrity of frozen-thawed epididymal and ejaculated spermatozoa were statistically similar, while the mean numbers of membrane-intact sperm bound to the zona of each oocyte were significantly different (Table 4.13). These results complement the findings of Hewitt et al. (2001), which

indicated that a cryopreservation protocol similar to that used for ejaculated spermatozoa was suitable for freezing epididymal spermatozoa.

Similar to previous reports, sperm motility appeared to be a more sensitive indicator of freezing damage than membrane integrity in the present study. The mean percentages of sperm motility were lower than those of membrane integrity (Figures 4.4, 4.8, 4.12, and 4.16). The decrease in motility could be due to changes in active transport and permeability of the plasma membrane in the sperm tail region (Watson, 1981). Also, egg yolk appears to protect the plasma membrane from damage caused by membrane transitions caused by temperature changes but does not seem to protect sperm motility (Thomas et al., 1993; Watson, 1995).

Since significant differences in the freezing susceptibilities of the epididymal and ejaculated spermatozoa of individual dogs were found in all experiments (Figures 4.3, 4.7, 4.11, and 4.15), these results confirm previous studies showing male-to-male differences in freezing susceptibility of canine spermatozoa (Songsasen et al., 2002; Yu et al., 2002). Variations have also been noted among the freezing susceptibilities of spermatozoa of individual stallions, boars, and humans (Amann and Pickett, 1987; Cochran et al., 1983; Heuchel et al., 1983; Johnson et al., 1981; Larsson et al., 1976). Similar effects have been noted in the dog by another laboratory (England, 1993). One possible explanation of male-to-male differences in spermatozoa survival may be that such differences reflect properties of sperm membranes that are genetically determined (Yu et al., 2002). Songsasen and Leibo (1997) reported that spermatozoa from three inbred strains of mice had significant differences in post-thaw survival. In another example, spermatozoa from a set of genetically

identical bulls exhibited similar post-thaw survival (Leibo and Bradley, 1999; Nishimura and Leibo, 1995).

Thurston et al. (2002) have identified molecular markers linked to genes controlling gene freezability. For individual boars previously classified as having spermatozoa that freeze either well or poorly, DNA was screened for amplified restriction fragment length polymorphism (AFLP) markers. Sixteen candidate genetic markers were identified by Thurston et al. (2002) by comparing the AFLP profile with spermatozoa freezability using logistic regression analysis. Also, breed influences have been noted in post-thaw fertility of boar semen (Johnson et al., 1981), and a similar relationship has been noted in the dog (Linde-Forsberg et al., 1999).

A second explanation of male-to-male differences in post-thaw sperm survival might be differences in permeability characteristics of their spermatozoa (Yu et al., 2002). De Leeuw et al. (1990) found differences between bull and boar spermatozoa in the distribution pattern of intramembranous particles on the sperm head membrane upon cooling. Phelps et al. (1999) reported a significant difference between permeability coefficients of spermatozoa from outbred mice and an inbred hybrid strain, which also suggests that membrane properties of spermatozoa could be genetically determined. A third explanation might be differences among males in proportions of spermatozoa with morphological abnormalities resulting from defects of sperm maturation (Yu et al., 2002). Morton and Bruce (1989) reported that canine spermatozoa that have been “decapitated” or that exhibit fractured necks, coiled or bent mid-pieces, or coiled tails are associated with poor post-thaw motility.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Preservation of spermatozoa is an important tool to assist in the reproductive success of a species. Endangered and threatened canid species may benefit from the use of semen preservation as a tool for assisting conservation (Goodrowe et al., 2000; Watson and Holt, 2001). The goal of this project was to derive a method by which canine epididymal spermatozoa can be frozen as small volumes at rapid rates on dry ice. To accomplish this, attempts were made to optimize the steps that influence the survival and fertilizing capacity of canine epididymal spermatozoa after being frozen as pellets on dry ice.

The first pregnancies from cryopreserved canine spermatozoa were produced over 30 years ago from semen frozen as pellets on dry ice (Seager, 1969), and artificial insemination of domestic bitches with frozen-thawed semen is now offered as a routine clinical service by veterinarians. However, resultant pregnancy rates are highly variable and are generally lower than those with fresh semen. There are many variables that apparently influence functional survival of cryopreserved dog spermatozoa. To complicate the derivation of methods of cryopreservation, spermatozoa from different individuals may exhibit significantly different responses to the same freezing treatments (Yu et al., 2002).

The overall purposes of this study were 1) to determine the effects of pellet volumes, cryoprotective agents, and thawing solutions on post-thaw survival of epididymal spermatozoa from individual dogs and 2) to determine the zona-binding capacity of epididymal spermatozoa from individual dogs prior to pellet-freezing and after thawing. Canine ejaculated spermatozoa were also

cryopreserved by the method found to produce the highest survival for epididymal spermatozoa to compare post-thaw survival and zona-binding capacity of the two categories of spermatozoa and to determine if the values for post-thaw survival of spermatozoa from an individual dog are reproducible.

The results of Experiment I indicate that canine epididymal spermatozoa exhibit maximum survival when frozen as pellets of 100 μ l volume on dry ice. The data also suggest the pellet volume affects cooling rate, which has been found to affect sperm survival (Yu et al., 2002). The results of Experiment II suggest that canine epididymal spermatozoa exhibit maximum survival when treated with the CPA glycerol and thawed in CCM or TALP.

In Experiments III and IV, the zona-binding capacity of epididymal spermatozoa from individual dogs decreased after pellet-freezing and thawing. Although zona-binding capacity varied greatly among dogs and did not correlate with motility or membrane integrity in this experiment, survival of frozen-thawed spermatozoa, as determined by all three assays, decreased compared to unfrozen spermatozoa. Using the particular cryopreservation method described in these experiments, the freezing and thawing of canine epididymal and ejaculated spermatozoa produced similar survival and zona-binding results. There was little variation in survival among ejaculates from an individual dog, which supports the assumption that the values for post-thaw survival of spermatozoa from an individual dog are reproducible. As previously reported, I found that sperm motility appeared to be a more sensitive indicator of freezing damage than membrane integrity. Since significant differences in the freezing susceptibilities of the epididymal and ejaculated spermatozoa of individual dogs were found in all experiments, these results confirm previous studies showing

male-to-male differences in freezing susceptibility of canine spermatozoa (Songsasen et al., 2002; Yu et al., 2002).

In conclusion, the overall goal of establishing a method by which canine epididymal spermatozoa can be frozen as small volumes at rapid rates on Dry Ice and yield reasonable survival was accomplished.

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APPENDIX

EFFECT OF AGE, CLINIC, AND TIME FROM SURGERY TO COLLECTION ON SURVIVAL OF SPERMATOZOA

Effect of age of dog, veterinary clinic from which a specimen was obtained, and time from surgery to collection on survival of spermatozoa.

Experiment	Assay Of Survival	Effect	Degrees of Freedom	F-Value (Variance Ratio)
I	motility	age	5	0.54
		time	1	0.18
		clinic	4	0.83
	membrane integrity	age	5	0.30
		time	1	0.09
		clinic	4	2.01
II	motility	age	4	0.47
		time	4	0.31
		clinic	4	0.89
	membrane integrity	age	4	0.54
		time	4	1.23
		clinic	4	0.71
III	motility	age	2	1.12
		time	2	0.72
		clinic	3	0.72
	membrane integrity	age	2	1.51
		time	2	1.77
		clinic	3	0.93
	zona-binding capacity	age	2	0.71
		time	2	0.13
		clinic	3	0.75
IV	motility	time	2	0.24
		clinic	2	1.05
	membrane integrity	time	2	0.22
		clinic	2	0.30
	zona-binding capacity	time	2	0.75
		clinic	2	0.99

P>0.05 for all F values shown.

VITA

Brooke Fahrig was born on December 5, 1978, in New Orleans, Louisiana. Brooke graduated from Hahnville High School as Salutatorian of her class in May 1996. She graduated *magna cum laude* from Louisiana State University in May 2000 with a Bachelor of Science degree in microbiology and a minor in speech communication. She attended medical school at Louisiana State University School of Medicine in New Orleans for a brief period beginning in August 2000 but was unhappy with that career choice and decided to discontinue her pursuit of a medical career.

After completing an internship at the Audubon Center for Research of Endangered Species in February 2001, Brooke worked as a Veterinary Technician and as a Zoo Keeper. She is a candidate for a Master of Science degree in Animal Science, Reproductive Physiology at Louisiana State University in Baton Rouge, Louisiana and the Audubon Center for Research of Endangered Species in New Orleans, Louisiana, under the supervision of Dr. Robert Godke and Dr. Stanley Leibo.